REMARKS

Claims 41-46, 50, 51 are currently pending in this application.

Applicants have amended claim 41 to include the term "transfer" before the term "vector" in the preamble and addition of the term "wherein said transfer vector transfers the defined nucleotide sequence into the nucleus of a cell." This amendment is supported, at least, on page 1. lines 2-4, on page 4, lines 3-2 and line 23, of the translated specification.

In the Final Office Action, mailed October 10, 2006, the Office maintained the rejection of claims 41-51 under 35 U.S.C. § 102 as being anticipated by either WO 97/12622 ("Verma") or Parolin et al., "Analysis in Human Immunodeficiency Virus Type 1 Vectors of *cis*-Acting Sequences That Affect Gene Transfer into Human Lymphocytes," *J. Virol.*, vol. 68, pp. 3888-95 (1994) ("Parolin"). These references, though, do not anticipate the claimed invention for the following reasons.

Verma

The Office maintained the rejection under § 102 in light of Verma because it asserted that Verma teaches "a recombinant vector comprising a polynucleotide - the HIV *pol* vector - that does include cPPT and CTS regions." Office Action mailed October 10, 2006, at page 3. Verma, though, does not teach a "transfer vector" that comprises the cPPT and CTS sequences of pol that achieves "wherein said transfer vector achieves transfer of the transgene or sequence of interest into the nucleus of a cell and forms a cis-acting triplex," as provided in the claimed invention.

The Office is apparently referring to the "first vector" depicted in page 4, lines 22-28 and Figure 1, "Vector 2" of Verma because this vector includes *pol* sequences. But,

this vector is a packaging vector in Verma, not a transfer vector. A packaging vector comprises DNA sequences encoding most structural viral proteins needed to form infectious viral particles, including *pol*. In this context, though, *pol* is incorporated into the final viral particle as a *trans*-factor and so is not able to form the DNA triplex needed for nuclear transport. Instead, the cPPT and CTS sequences of *pol* must be *cis*-acting to form a DNA triplex as provided in independent claim 41.

In Verma, the actual "transfer vector" that provides the *cis*-acting viral sequences is the "third" vector as depicted on page 6, lines 24-29, and in Figure 1 - vector 1. This vector does not include *pol* sequence, though. Furthermore, this vector is not able to form a DNA triplex.

Because Verma does not disclose a "transfer vector" capable of achieving "transfer vector achieves transfer of the transgene or sequence of interest into the nucleus of a cell and forms a cis-acting triplex" it cannot anticipate the claimed invention. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. § 102 in light of Verma be withdrawn.

Parolin

The Office maintained the rejection under § 102 in light of Parolin because it asserted that "the Parolin vector *comprises* a *pol* polynucleotide, which naturally contains the cPPT and CTS regions." Office Action mailed October 10, 2006, at page 4. Applicants disagree with the Office's characterization of Parolin and accordingly, traverse this rejection. Despite the Office's assessment, Parolin does not disclose the complete *pol* sequence and does not disclose the cPPT and CTS sequences required for the claimed invention.

Parolin recites: "The v1864 RSN vector contains gag and pol sequences up to the MseI site (nucleotide 2200) and the v2731 RSN vector contains gag and pol sequences up to the PflmI site (nucleotide 3067)." Parolin at p. 3889. Exhibit A is provided to demonstrate the position of the closest nucleotides recited in Parolin, at page 20, and the beginning of the cPPT and CTS sequences of the claimed invention, at page 26. As shown, nucleotides 2200 and 3067, which are the end-points of the *pol* sequences in the vectors v1864 RSN and v2731 RSN, respectively, disclosed in Parolin are upstream of the cPPT sequence.

Specifically, cPPT is located at the end of the *pol* sequence, inside the integrase sequence, as described in Charneau *et al.* 1991 (Exhibit B) at Figure 1. It should be noted that Charneau 1991 relies on numbering based on the DNA, beginning at the R element from the LTR 5' end, while Parolin relies on numbering based on the RNA beginning at the LTR5' end. This difference results in a shift of 421 base pairs between the numbering. Taking into account this shift, cPPT begins at position 4783, which corresponds to position 4362 in the HXBc2 HIV-1 provirus, the sequence used in Parolin, *see* Parolin at p. 3889, and CTS ends at position 4902, which corresponds to 4481 in HXBc2. Thus, cPPT and CTS begin at 4362, considerably downstream of 3067, the point at which the *pol* sequences in Parolin end. Parolin, then, does not include cPPT or CTS.

Because Parolin does not disclose a transfer vector with the cPPT and CTS sequences, it does not anticipate the claimed invention. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. § 102(b) in light of Parolin be withdrawn.

Please grant any extensions of time required to enter this response and charge the fee to our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: March 9, 2007

By: 

Deborah Katz
Reg. No. 51,863
Telephone: (202) 408-4382
Facsimile: (202) 408-4400
E-mail: deborah.katz@finnegan.com

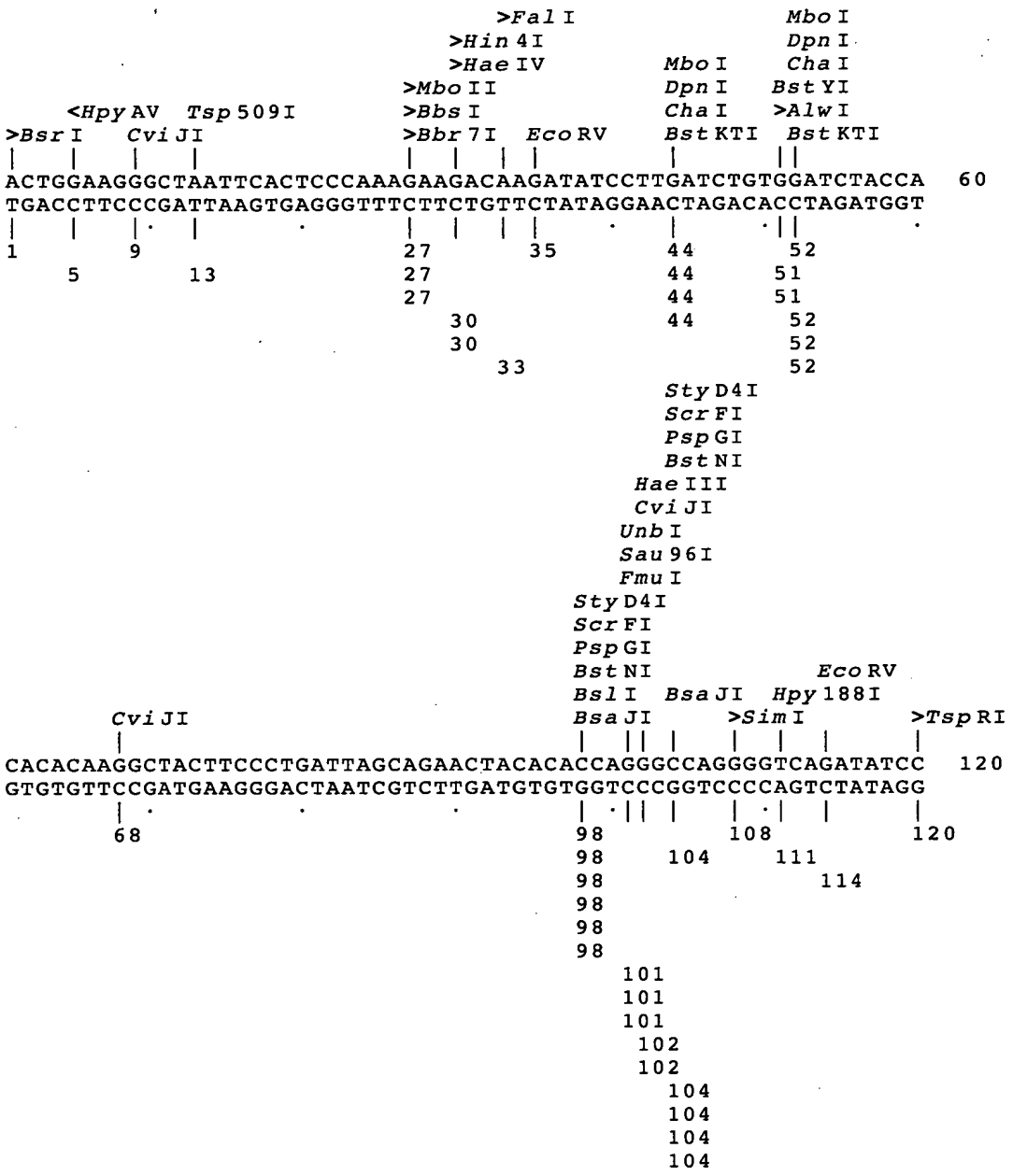
P. Charneau
Feb. 5, 2007

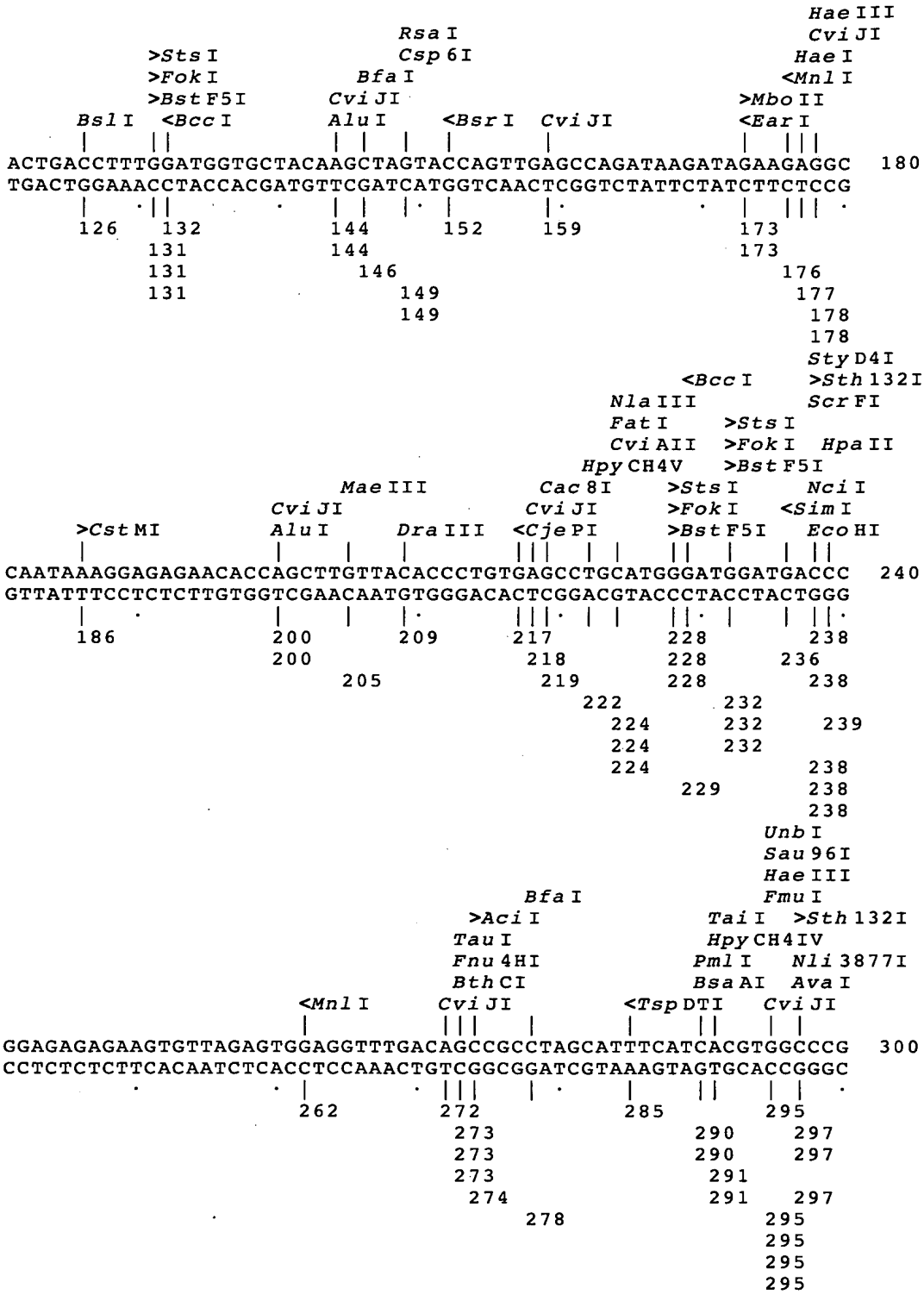
DNA Strider 1.4f1 ### lundi 5 février 2007 16:22:16 (US Letter @ 100%)

HXB2 -> Restriction Map

DNA sequence 9721 bp ACTGGAAGGGCT ... AAATCTCTAGCA linear

Positions of R. E. sites (sites unique in whole sequence are bold)

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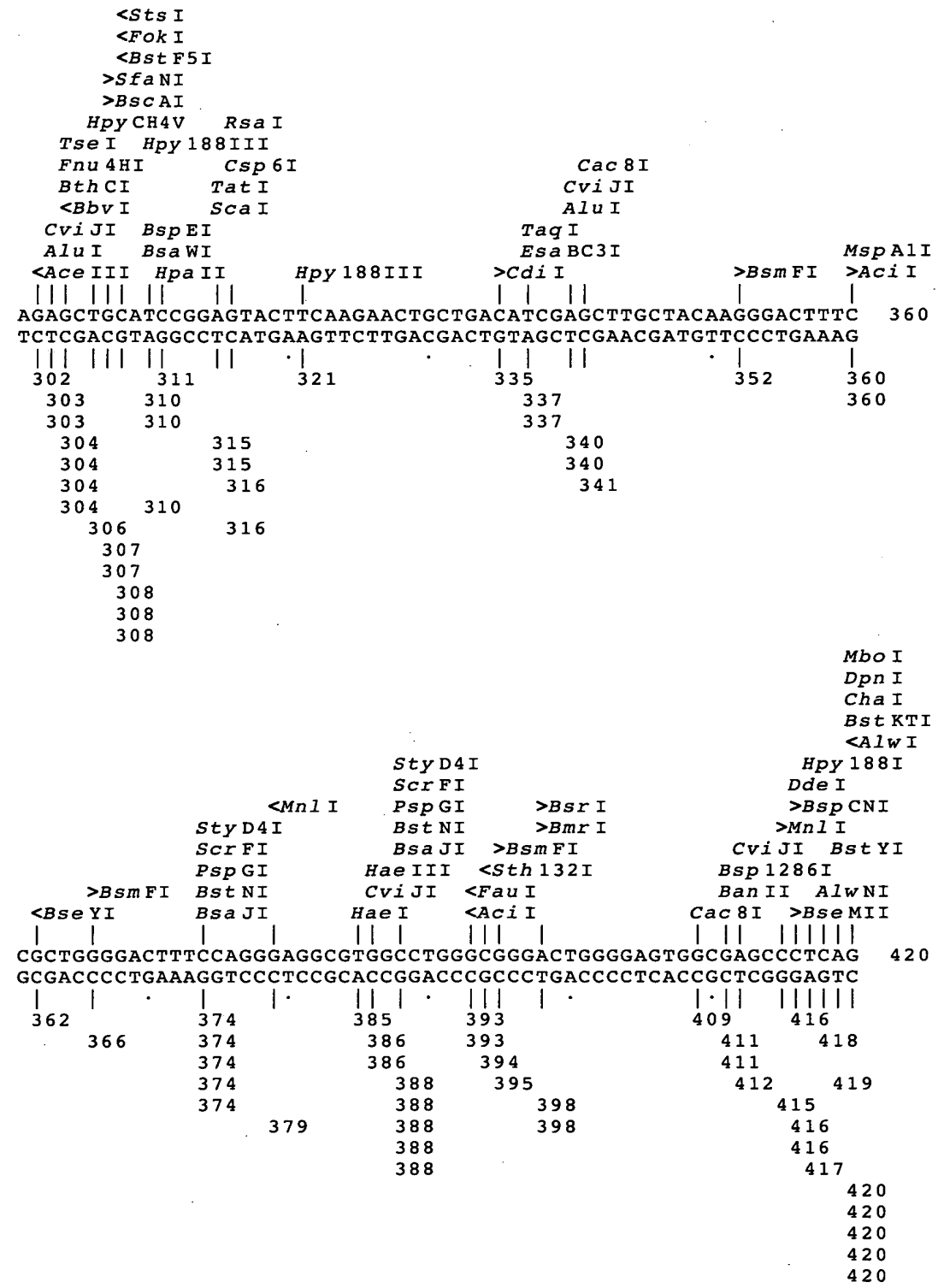
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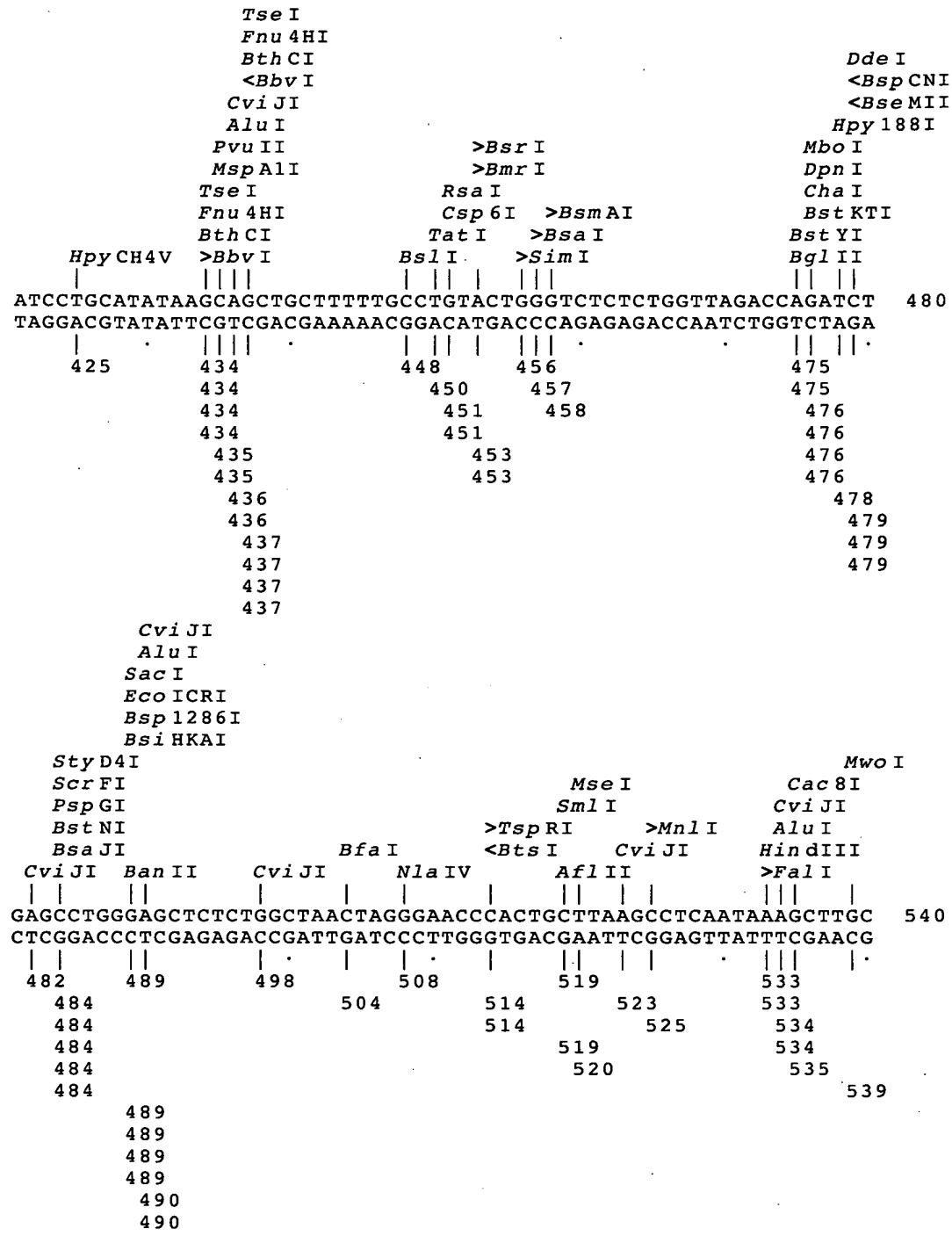
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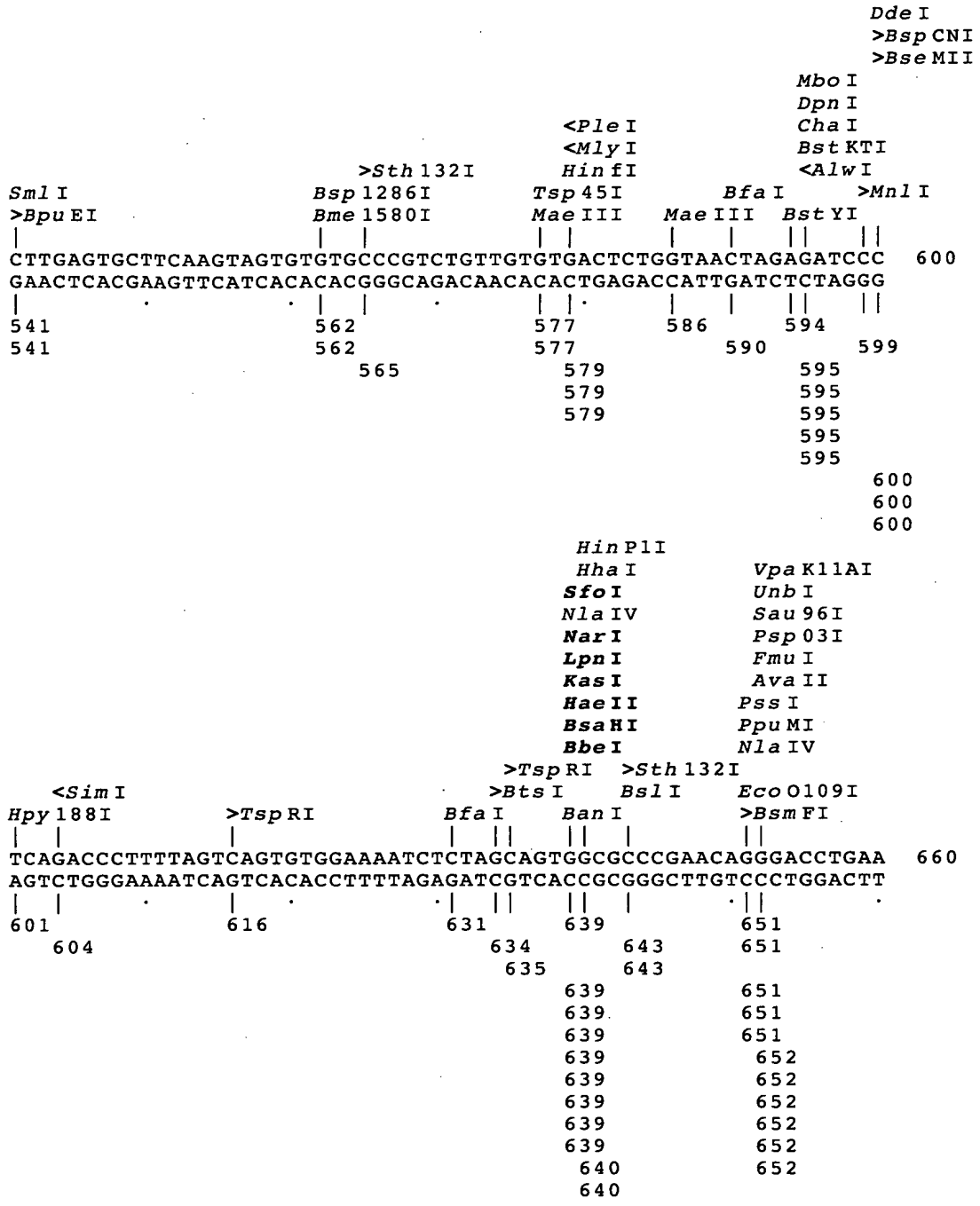
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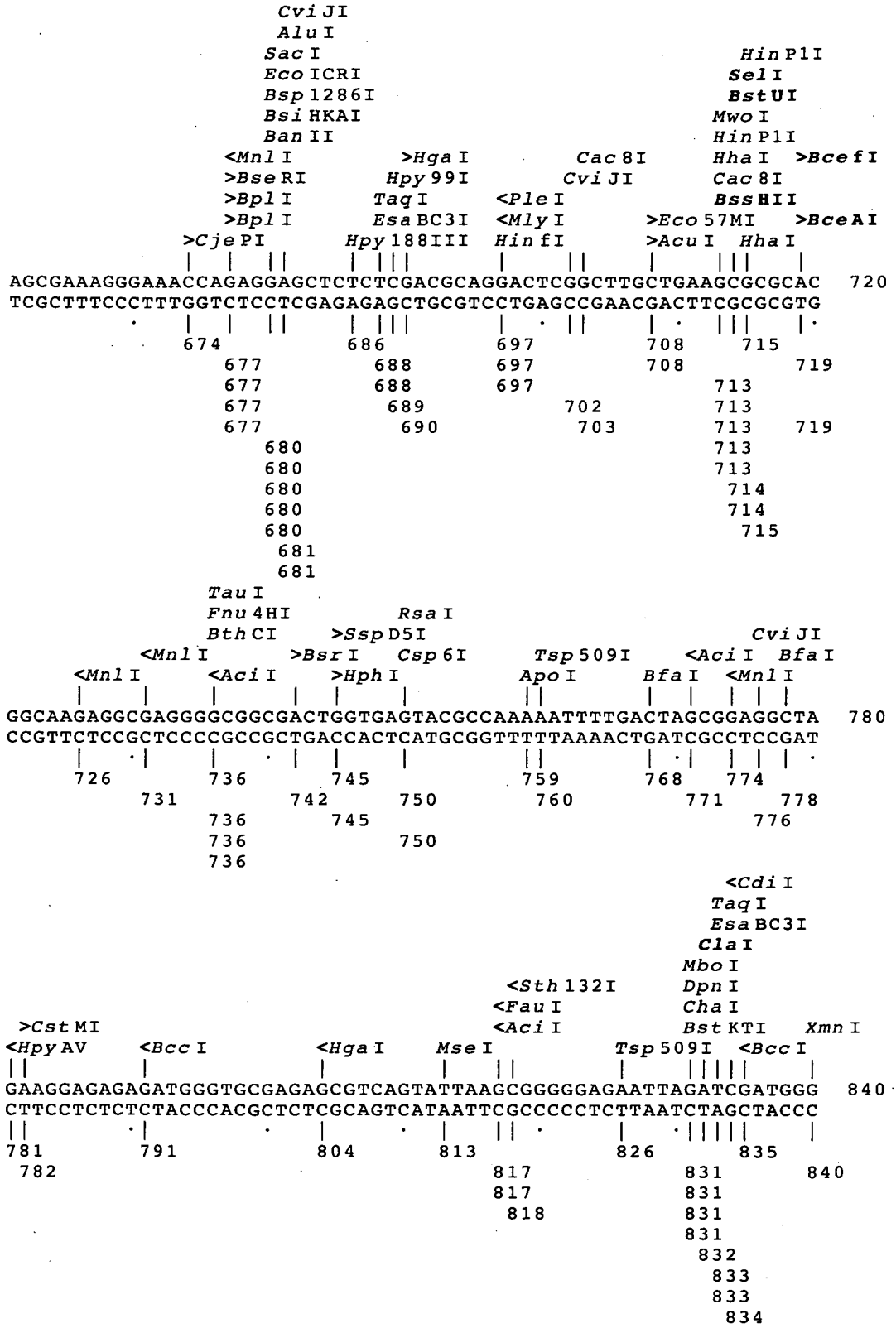
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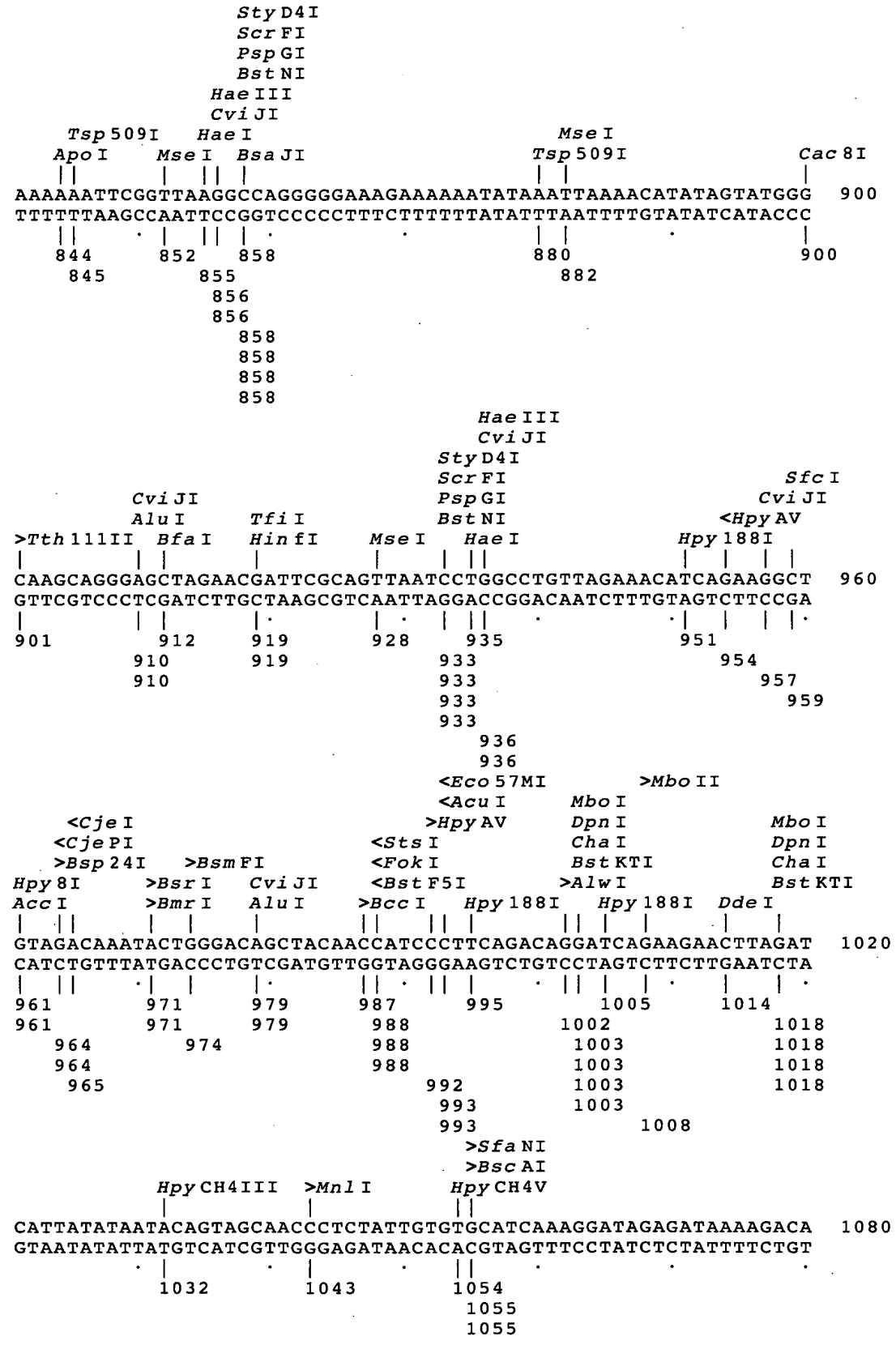
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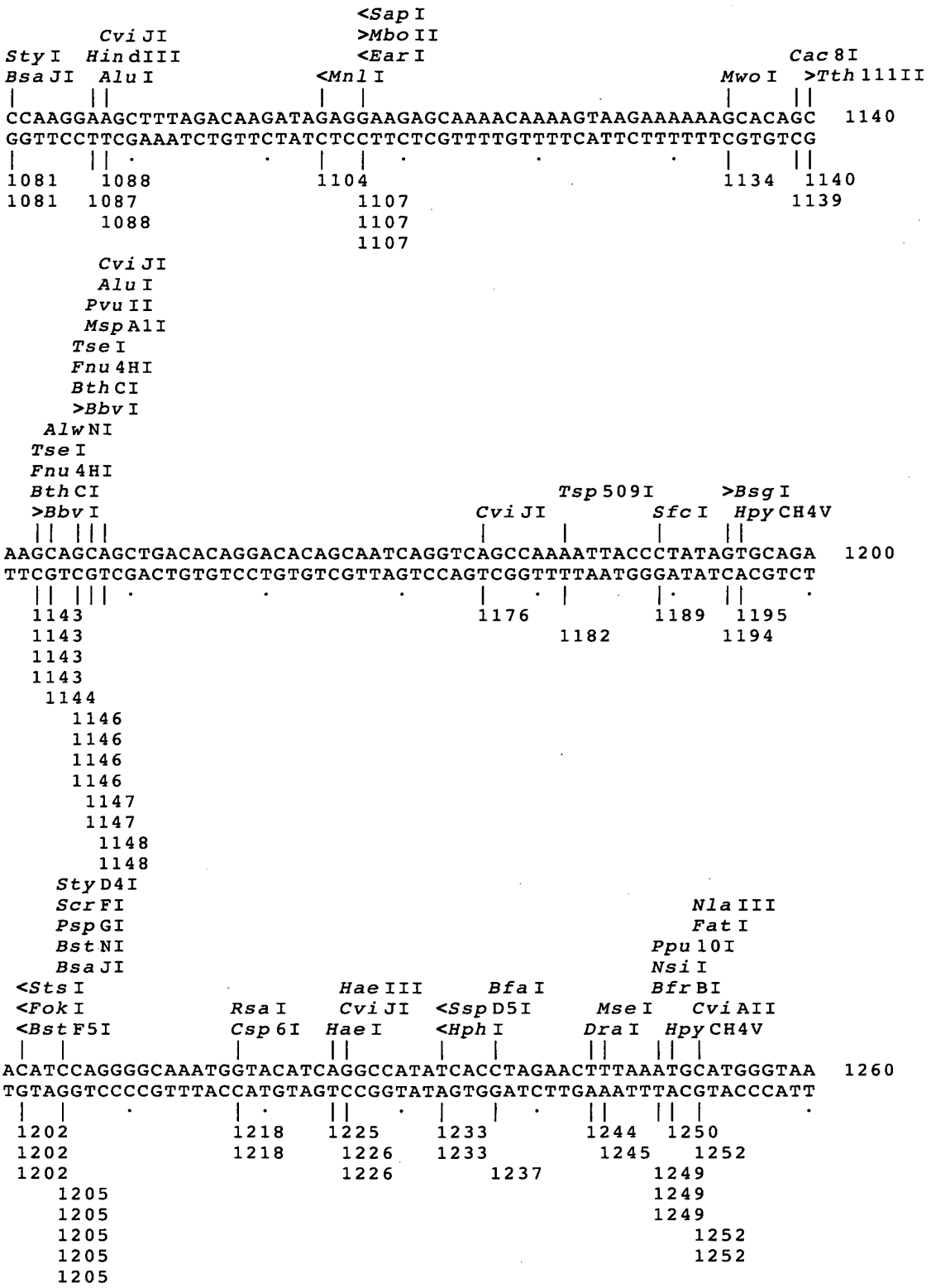
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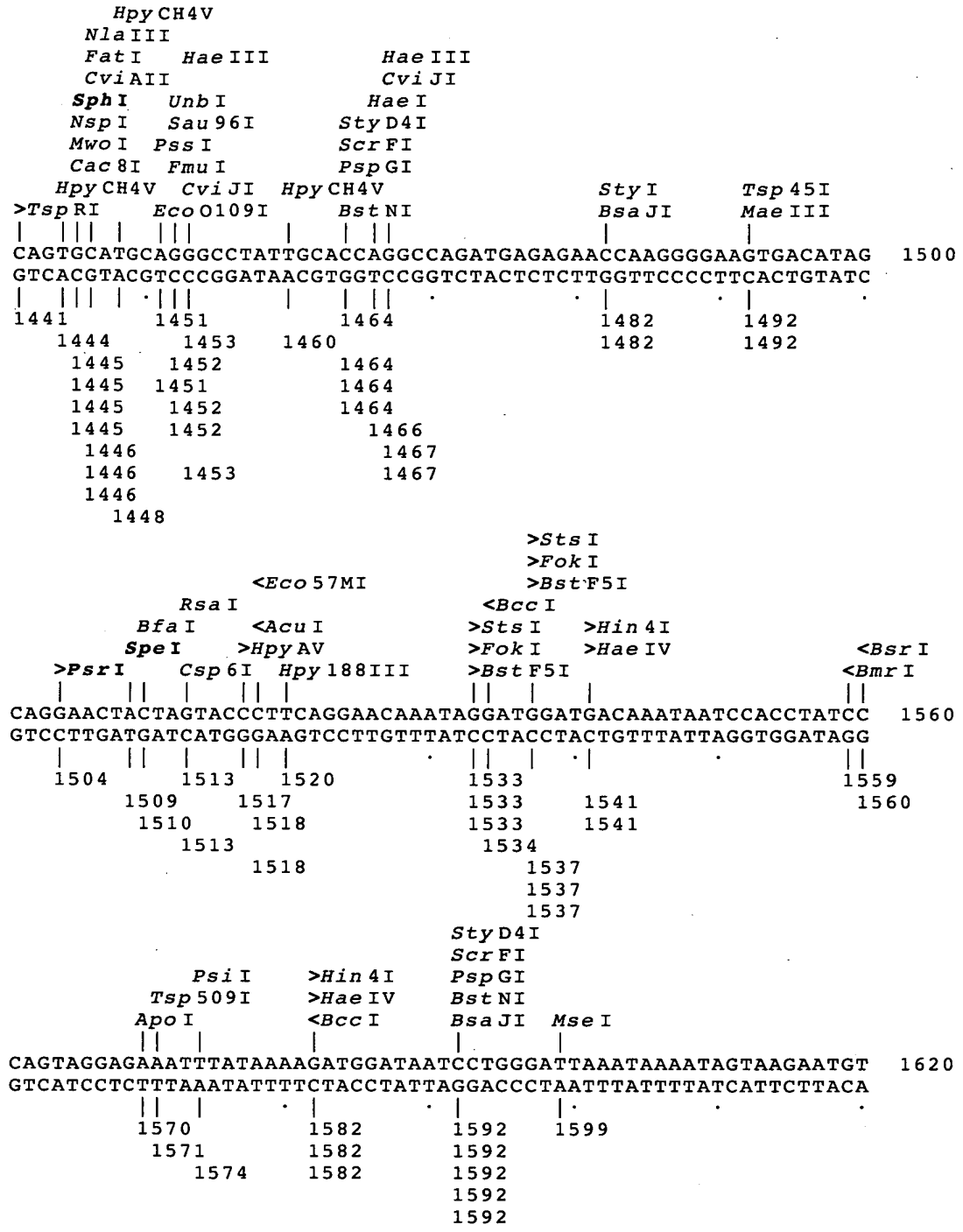
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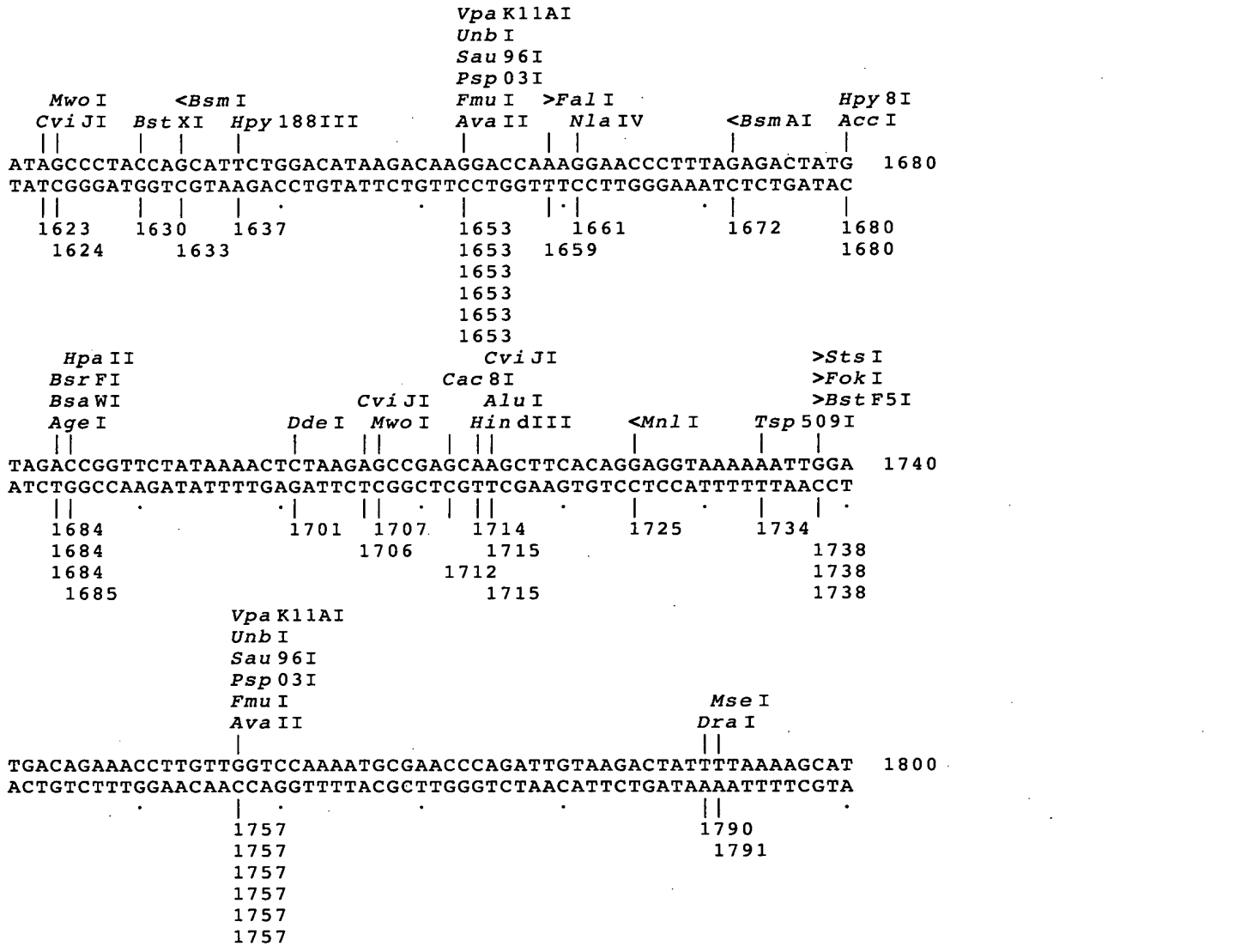
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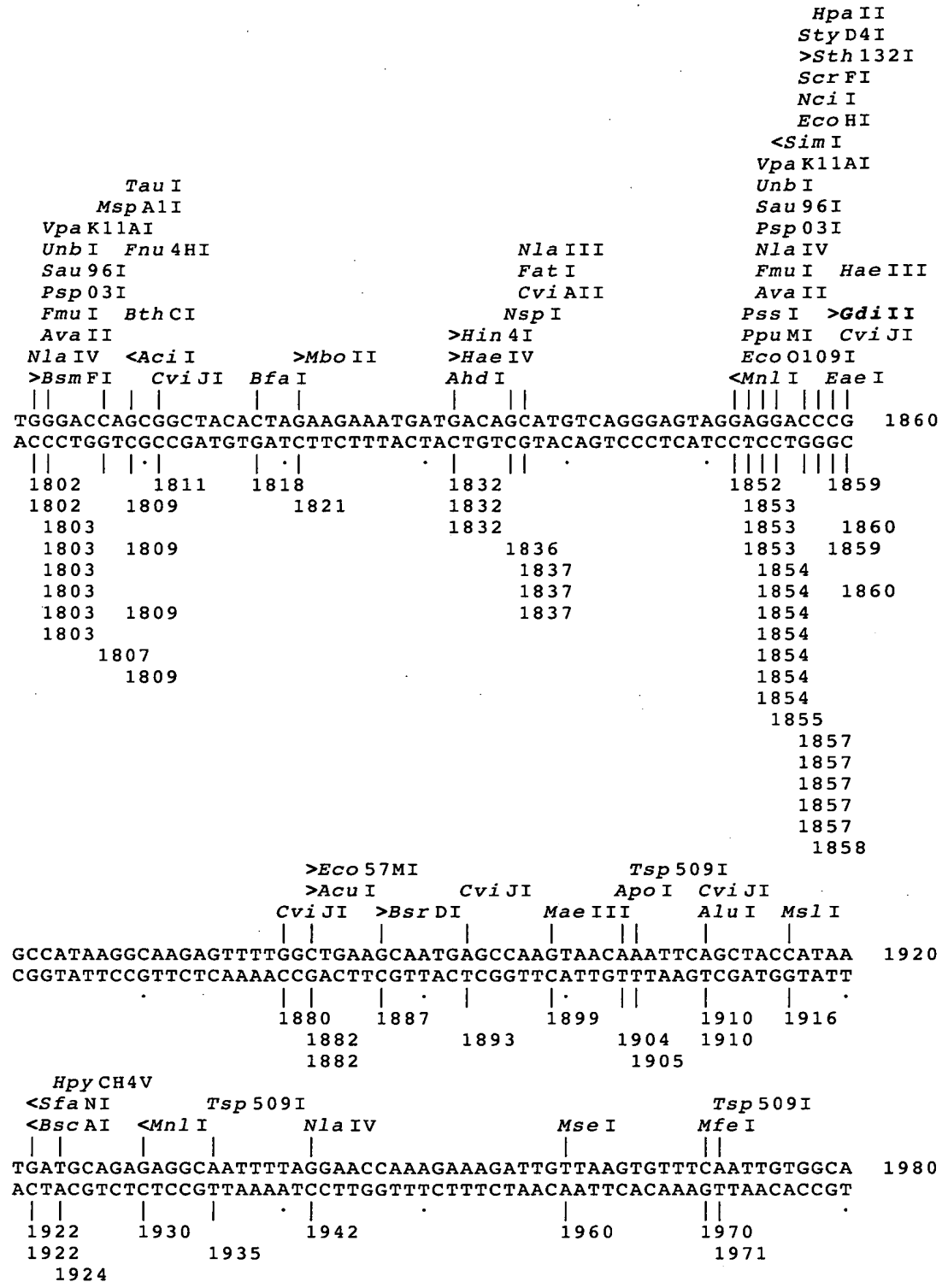
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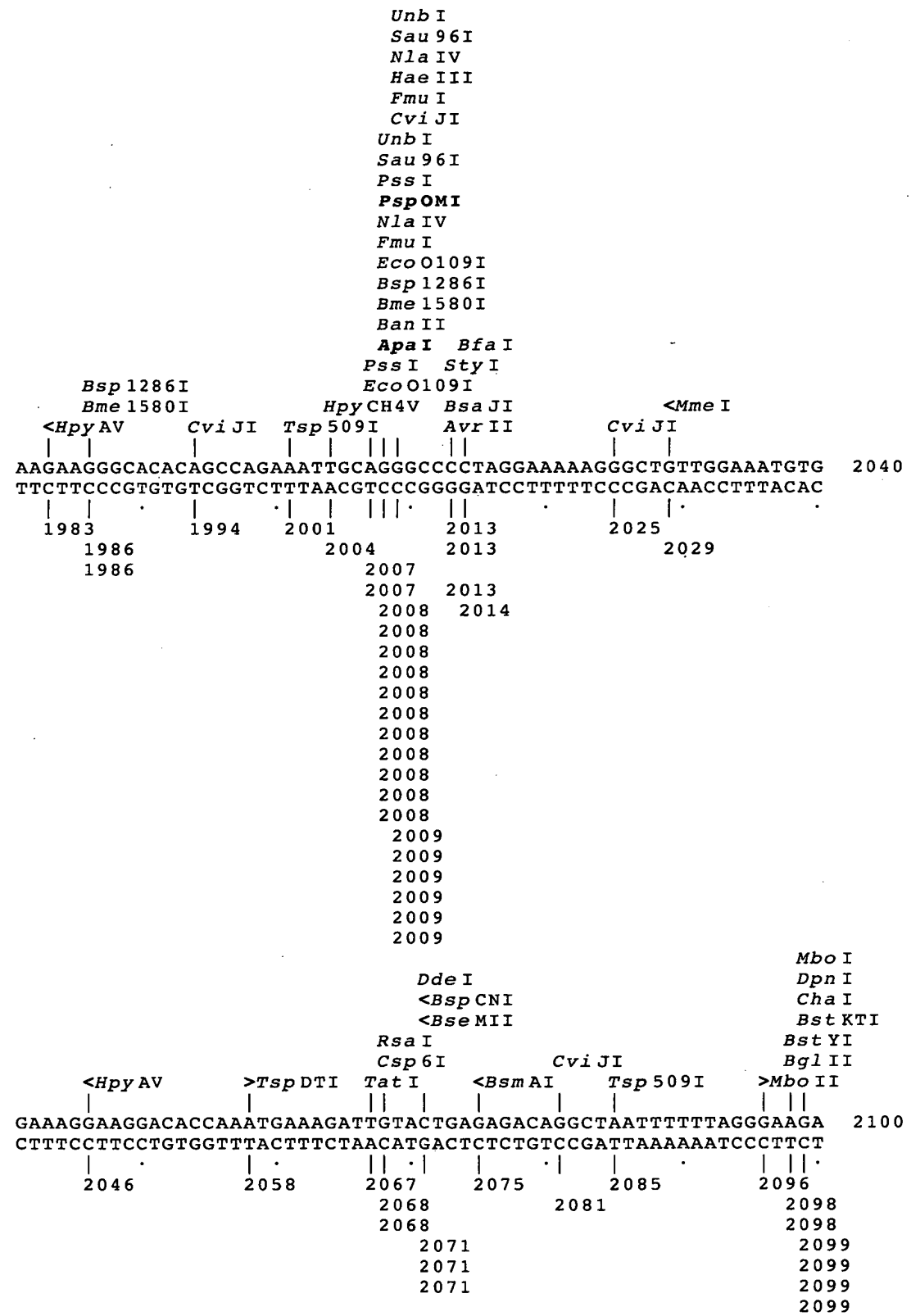
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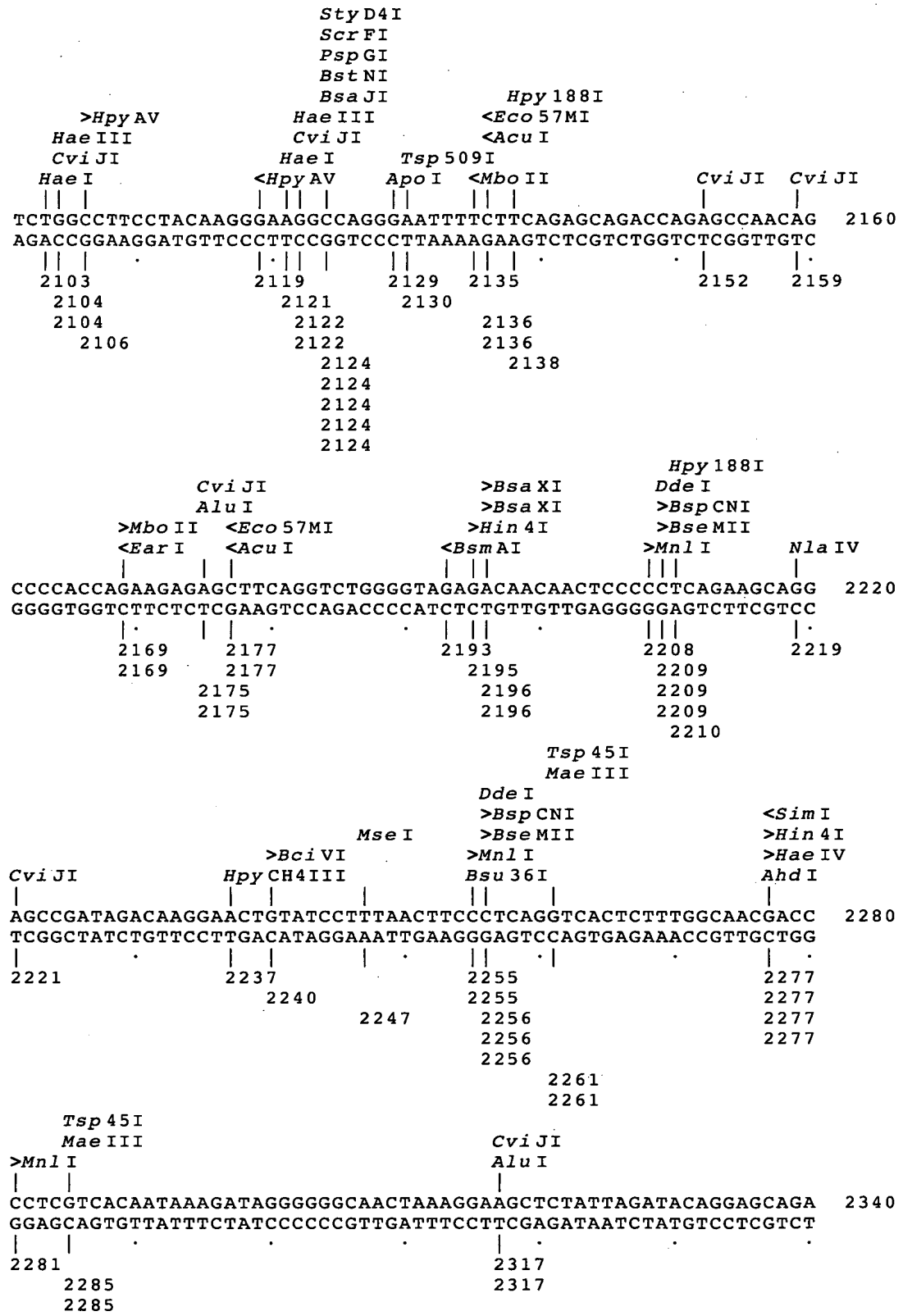
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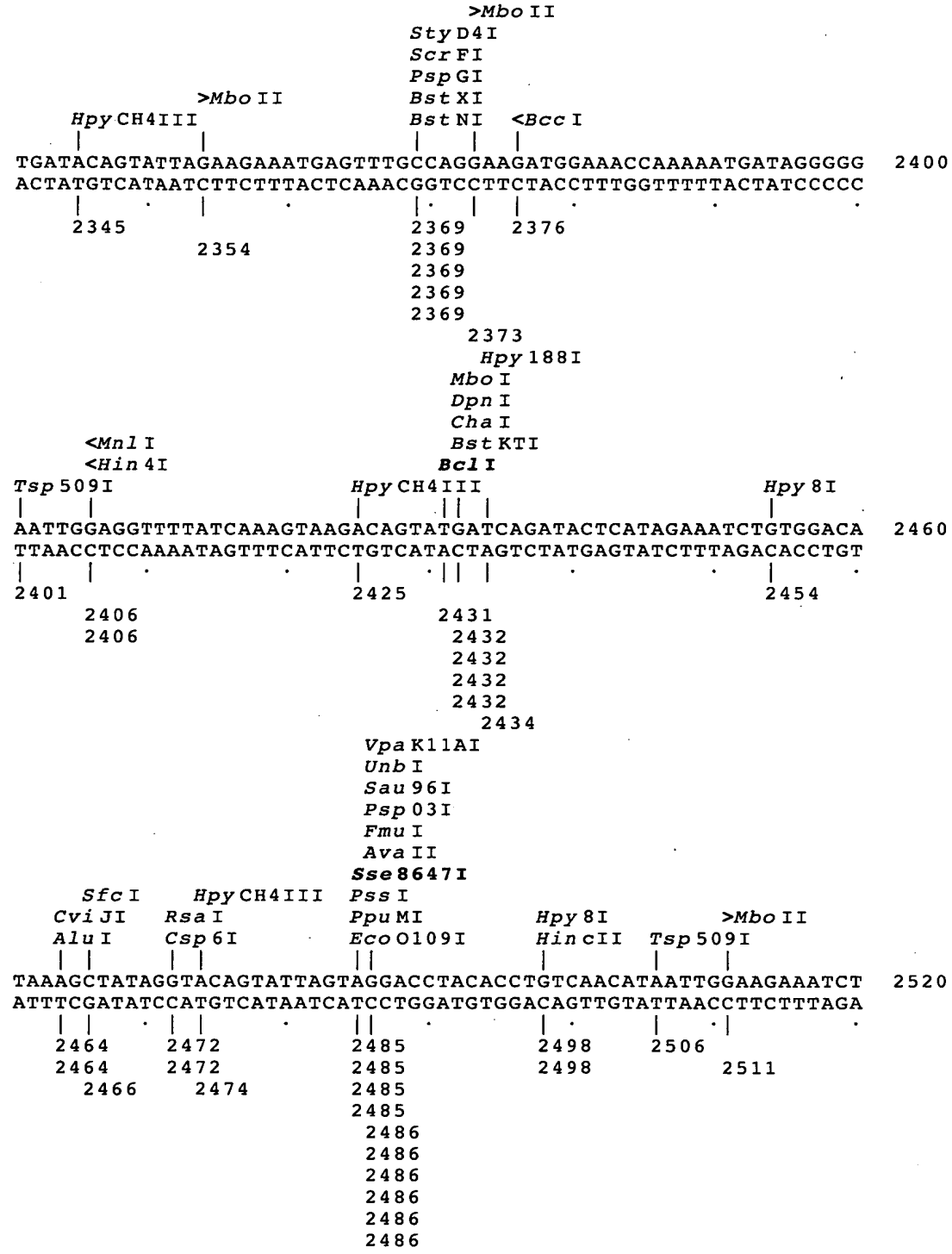
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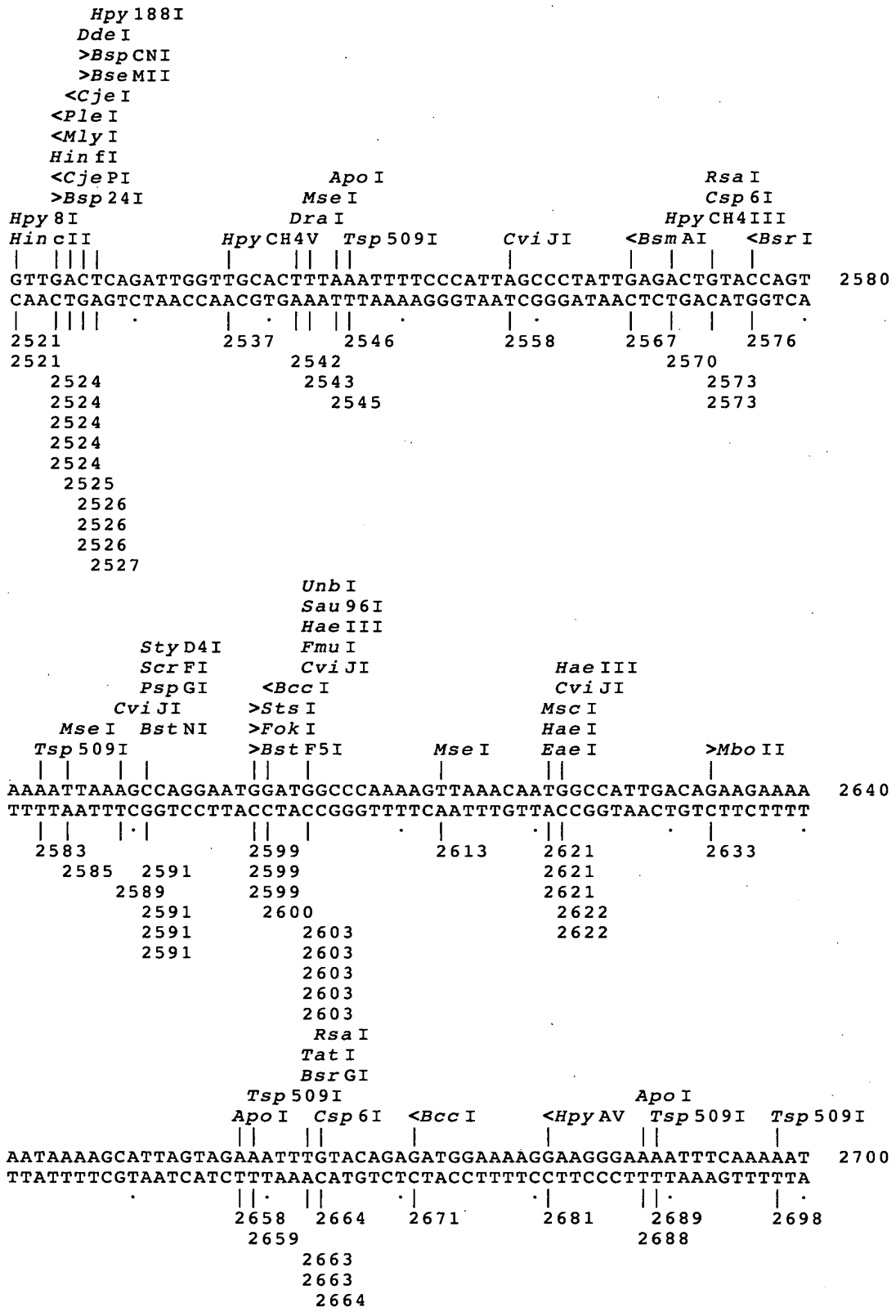
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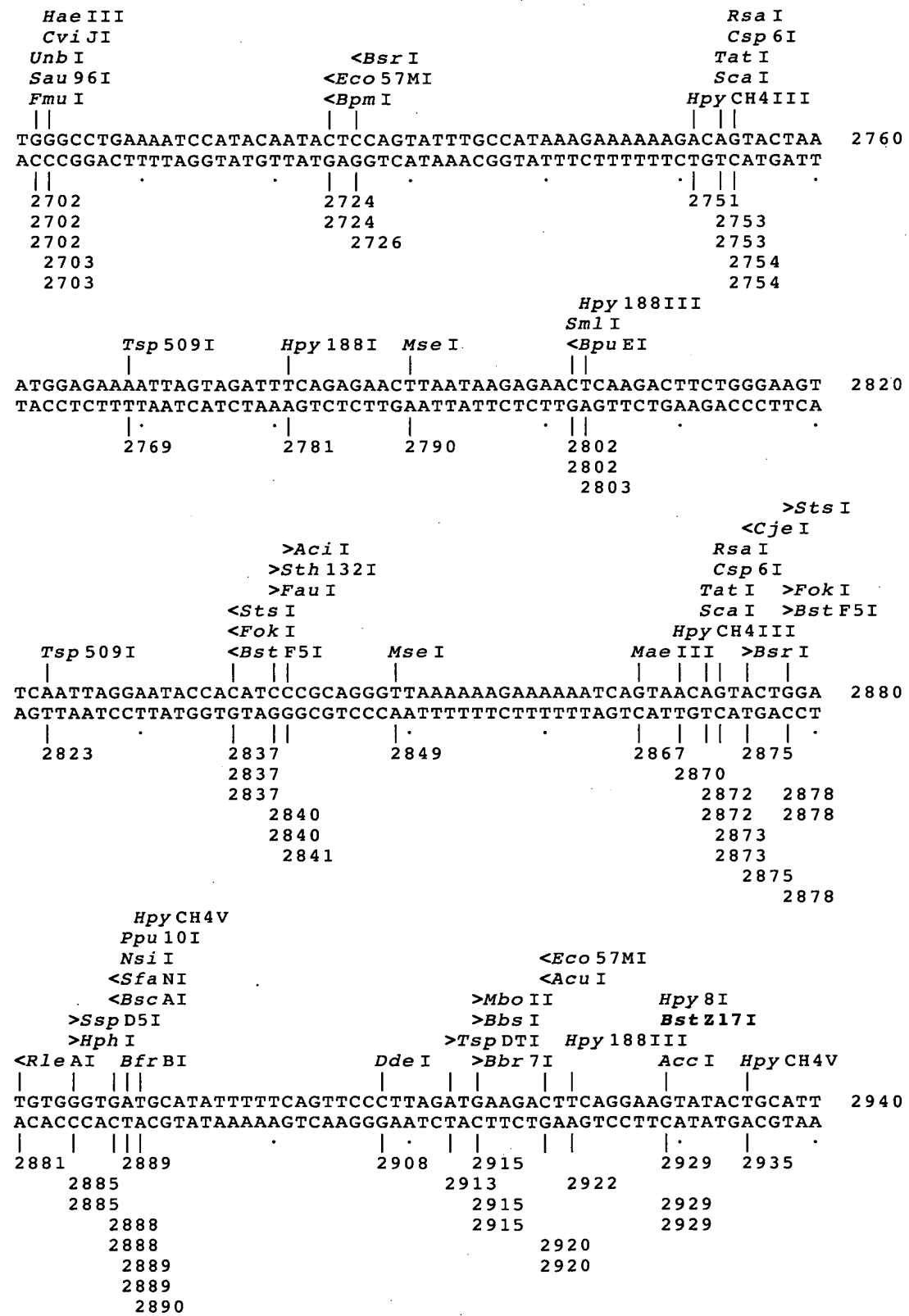
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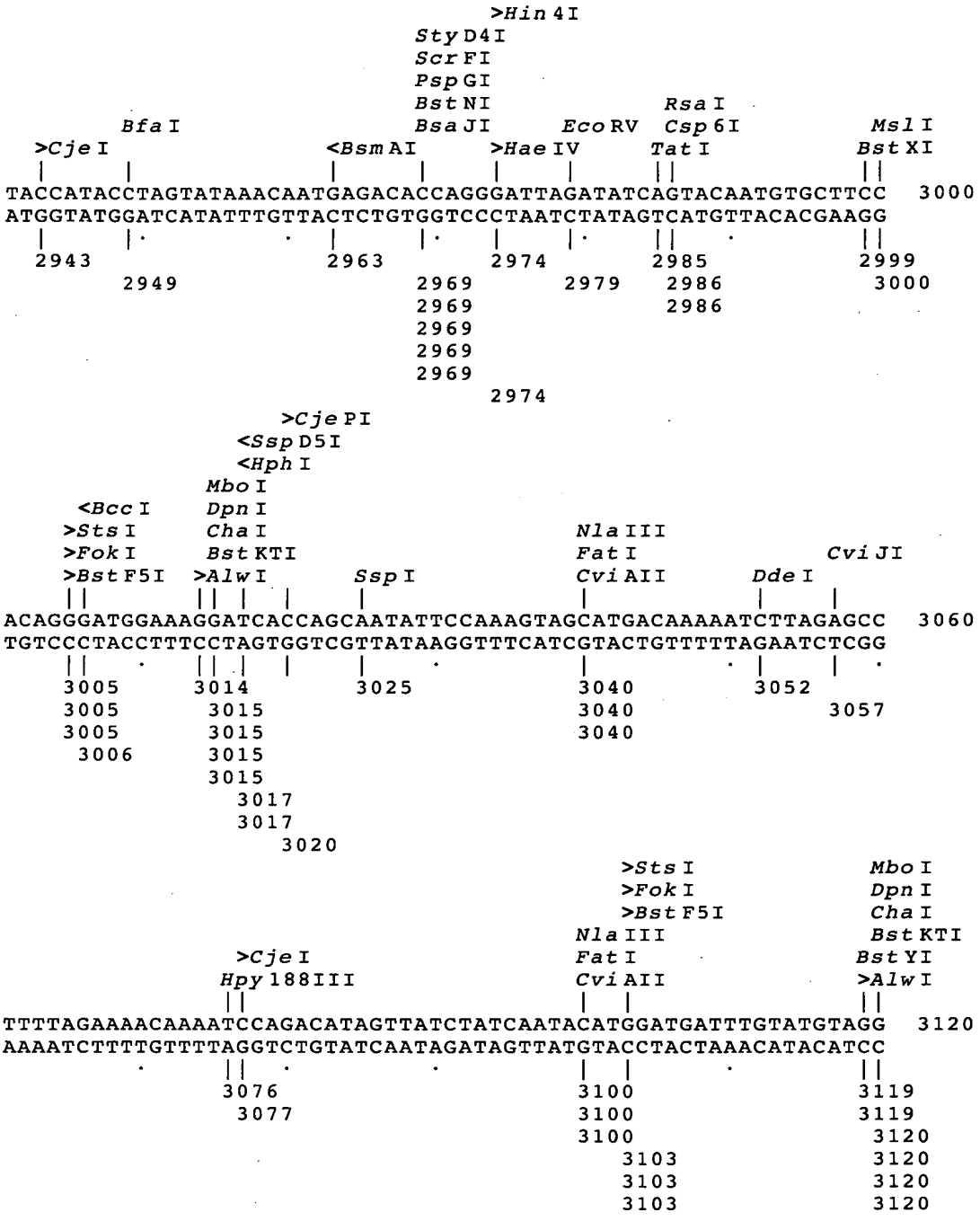
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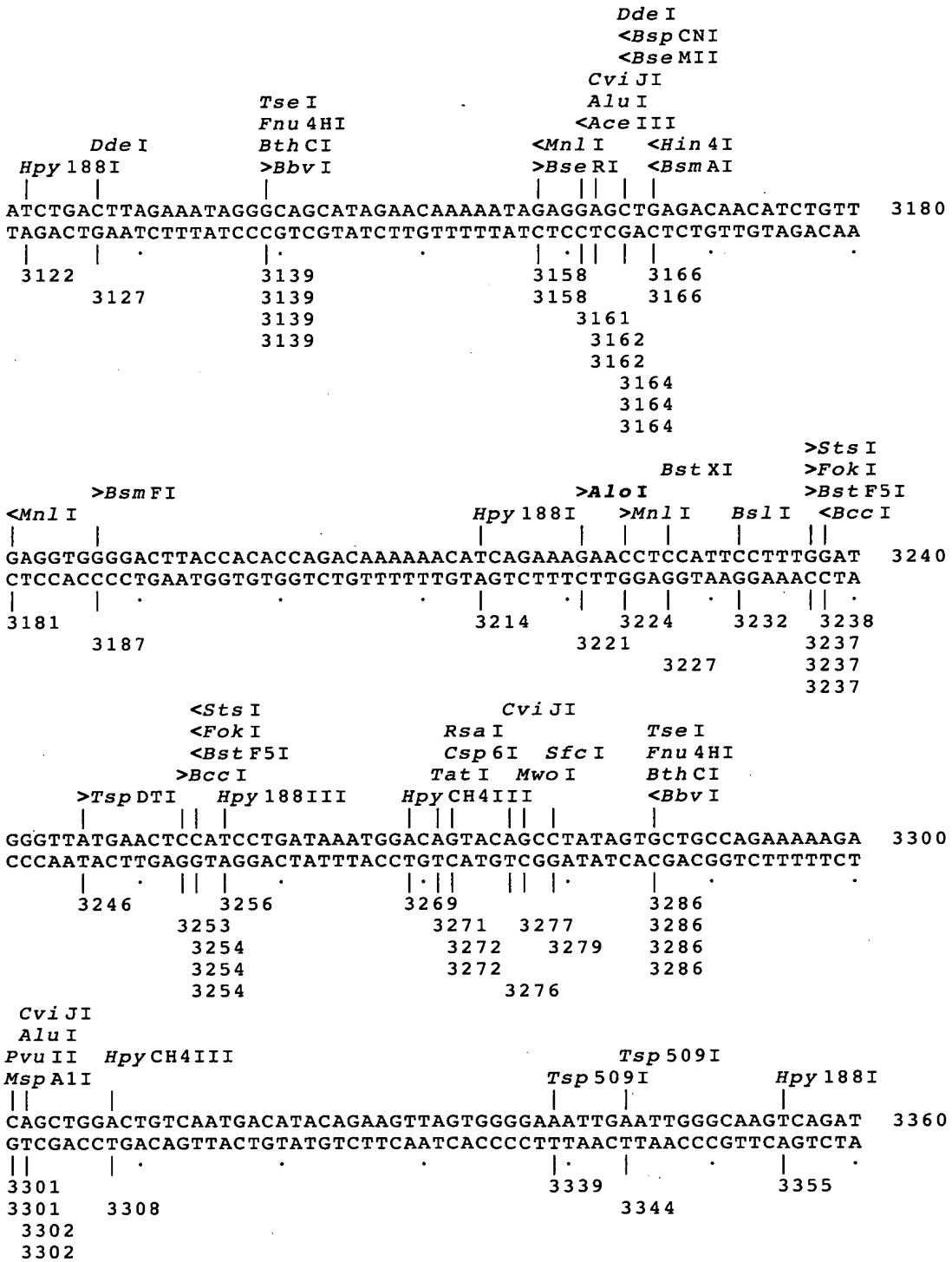
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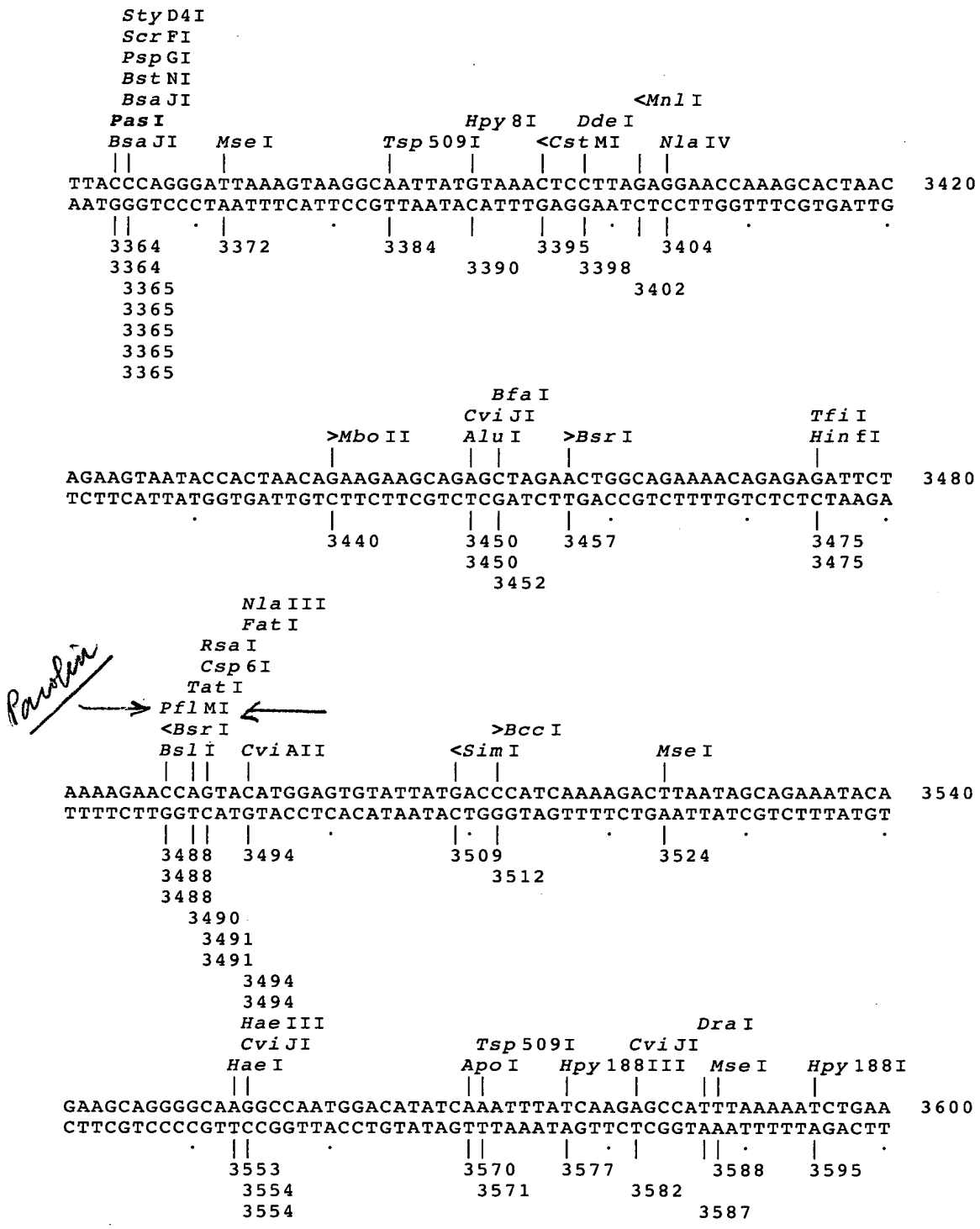
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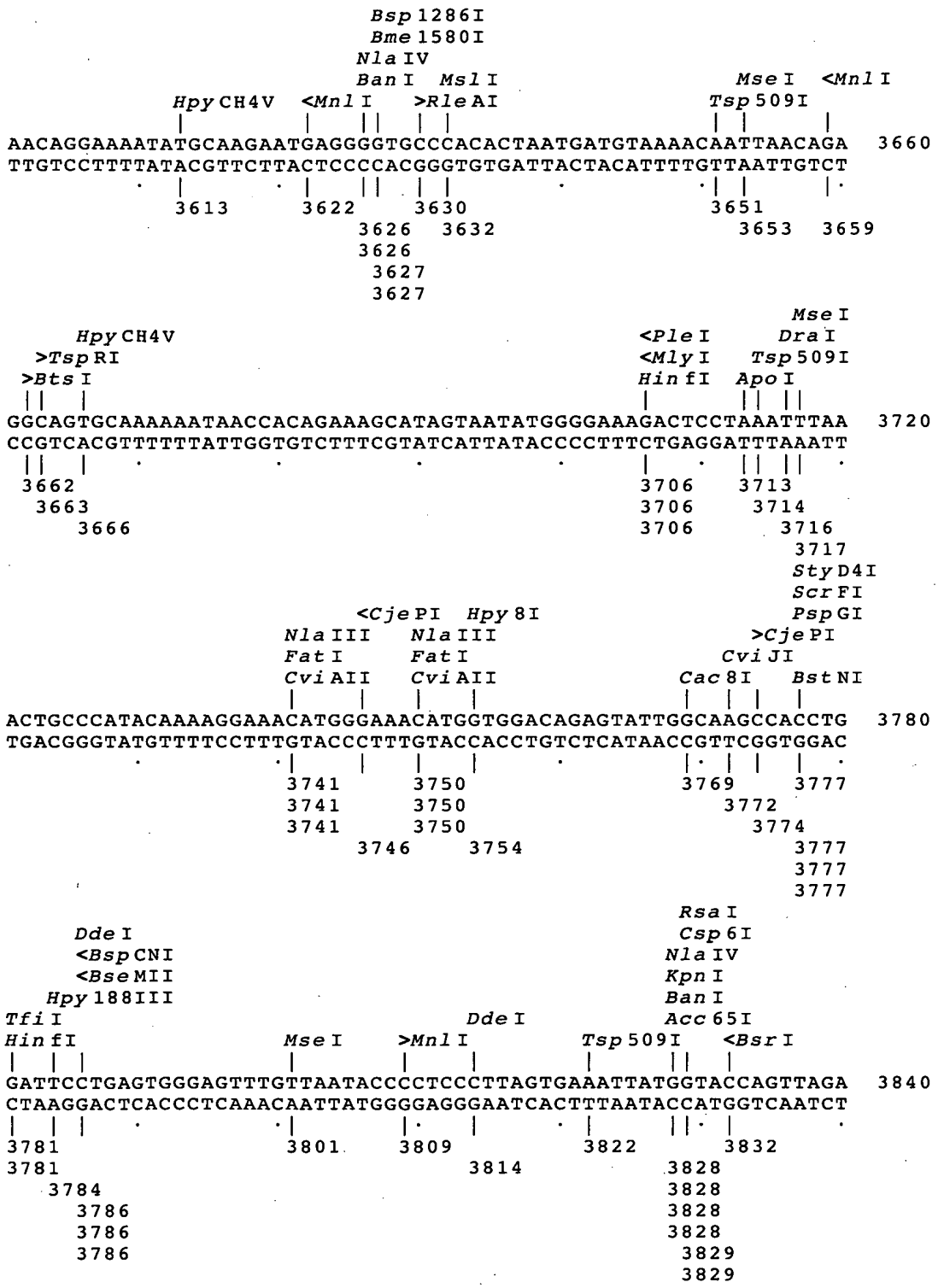
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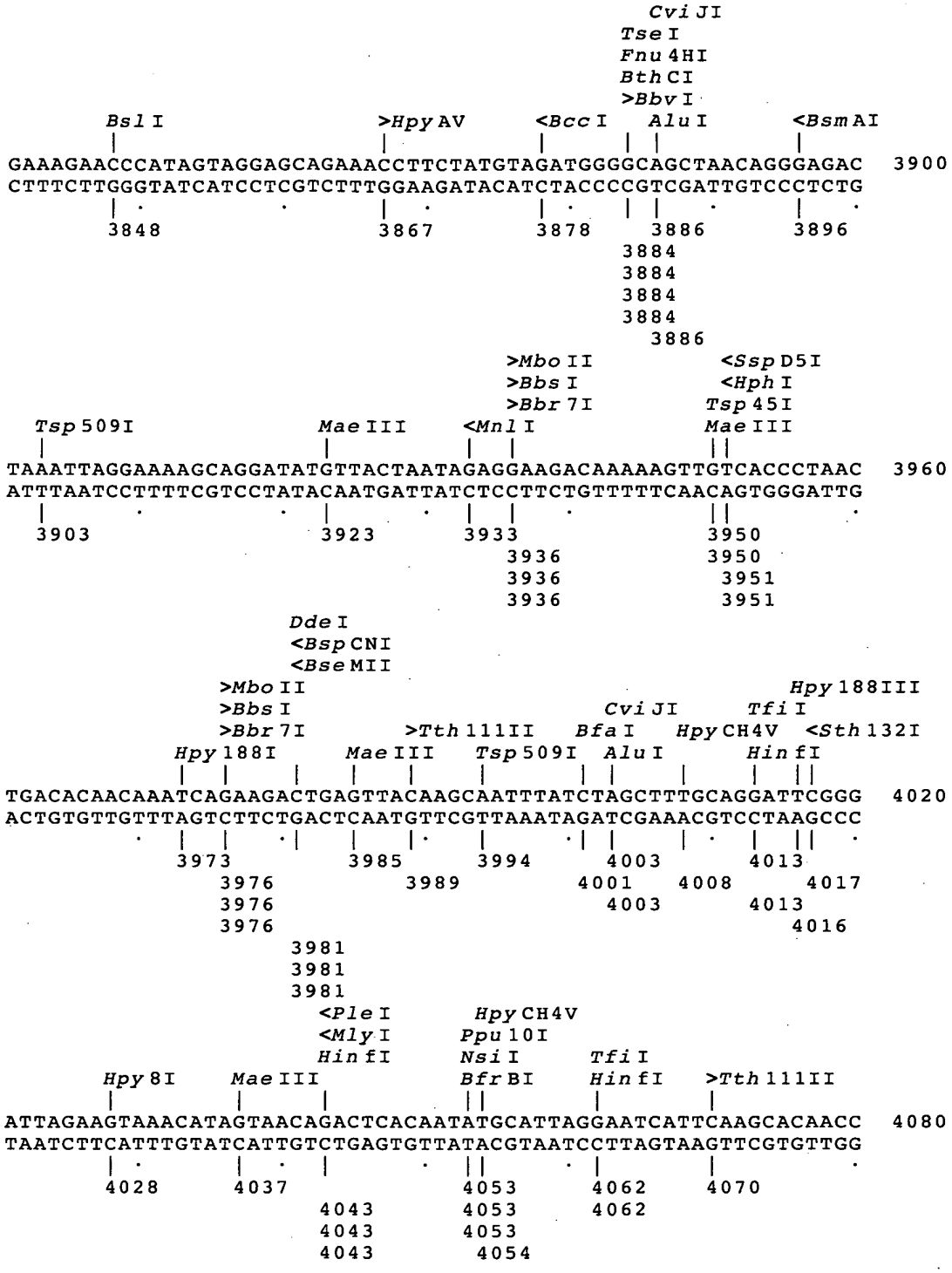
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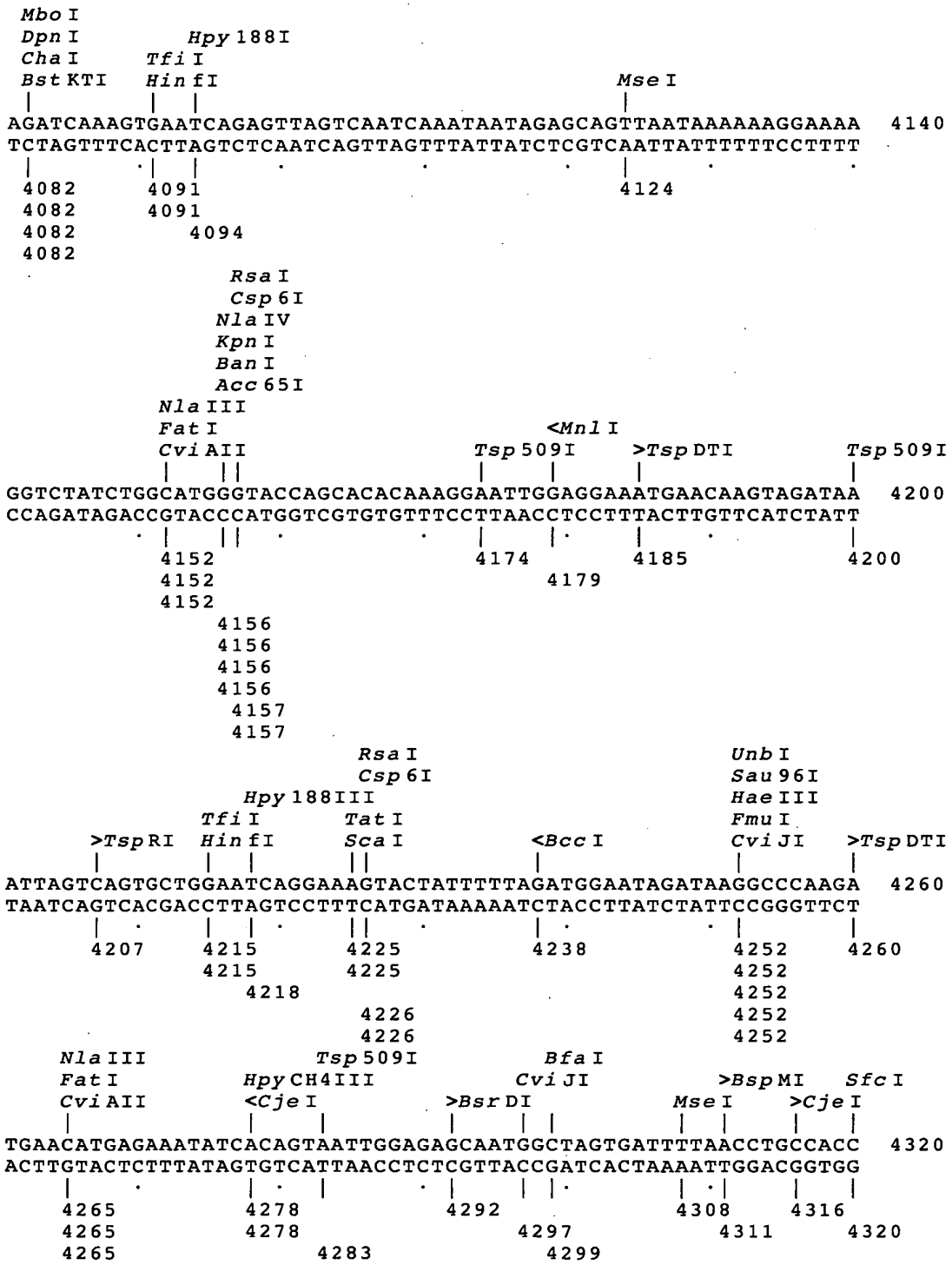
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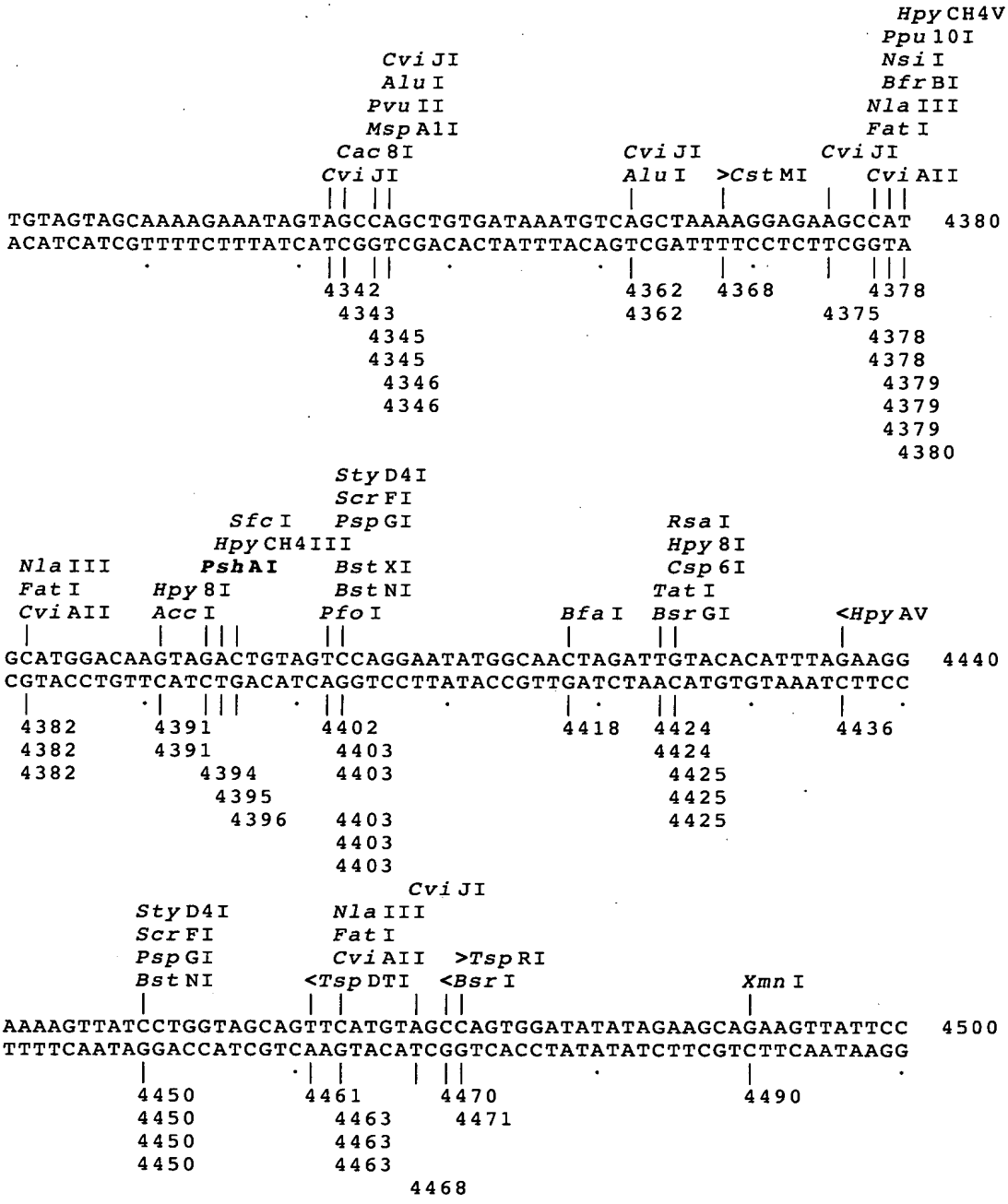
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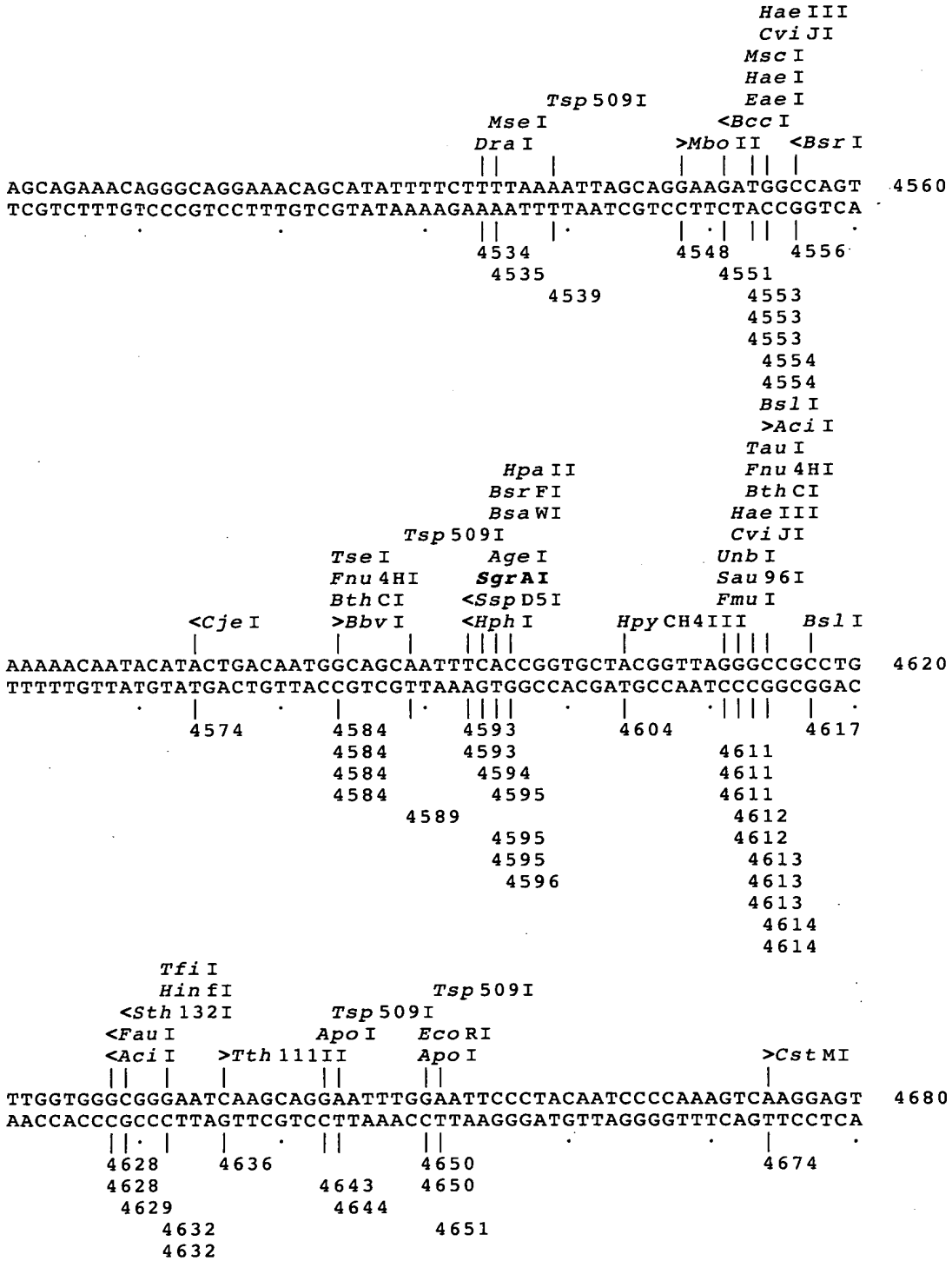
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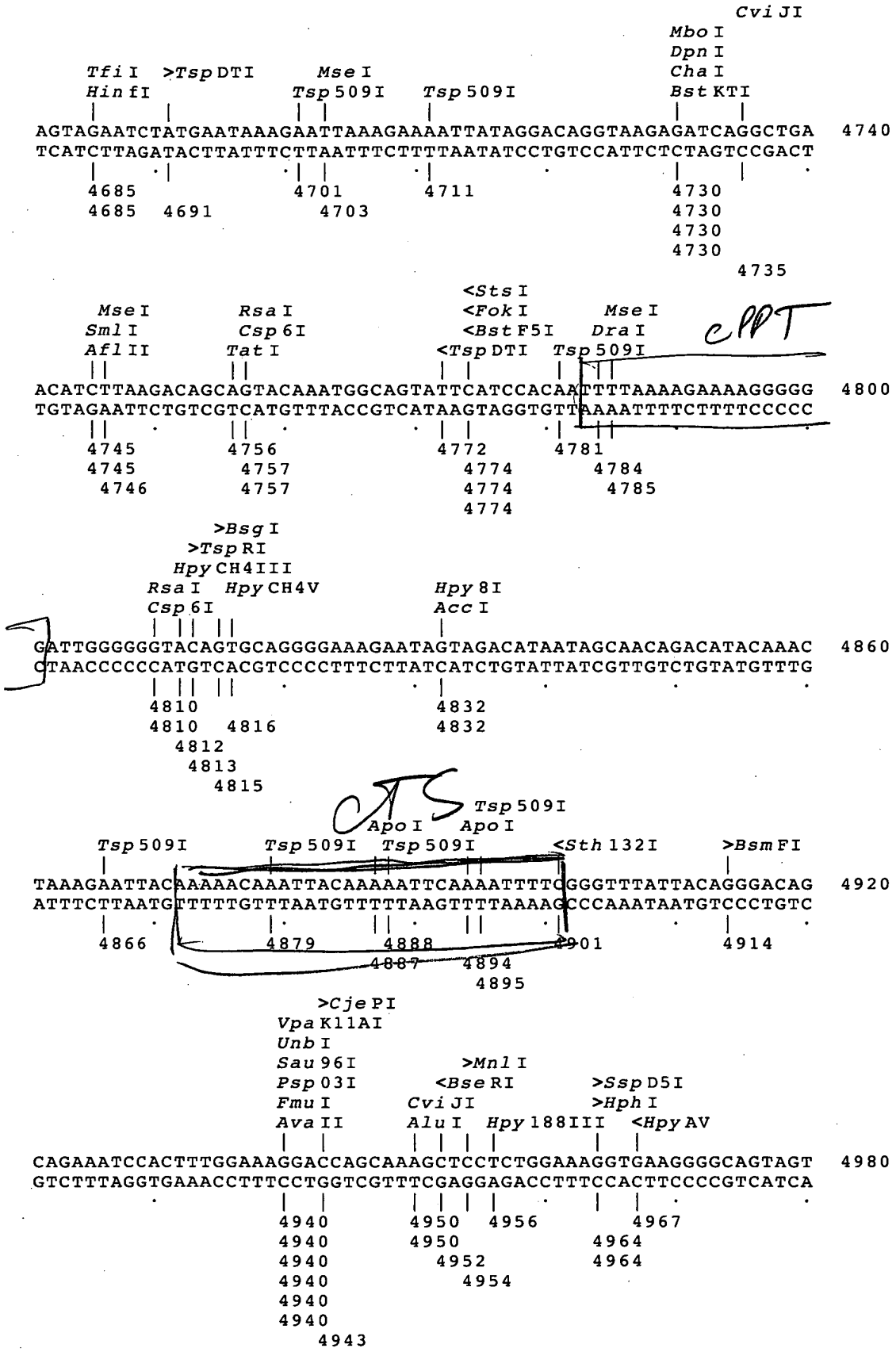
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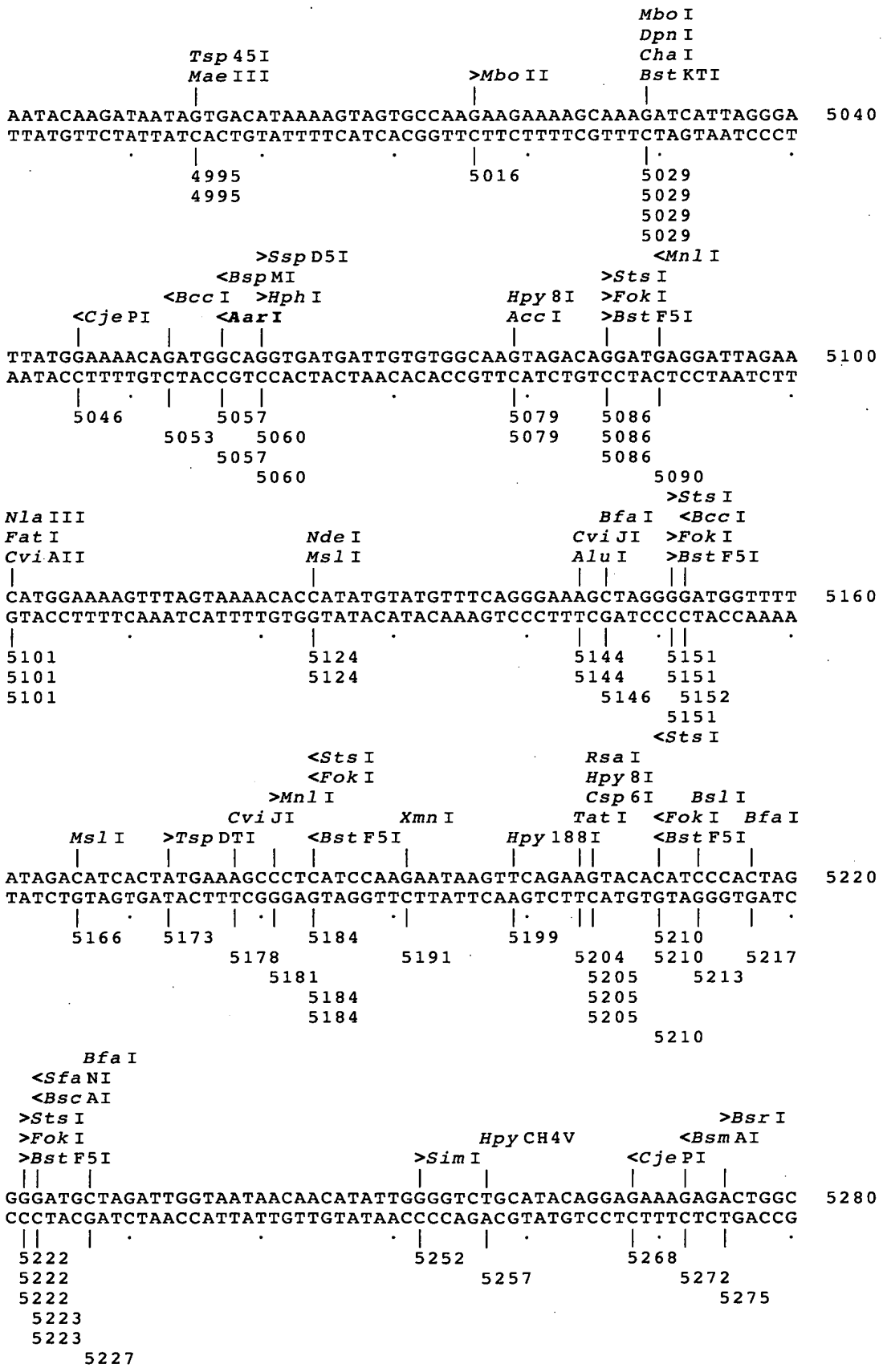
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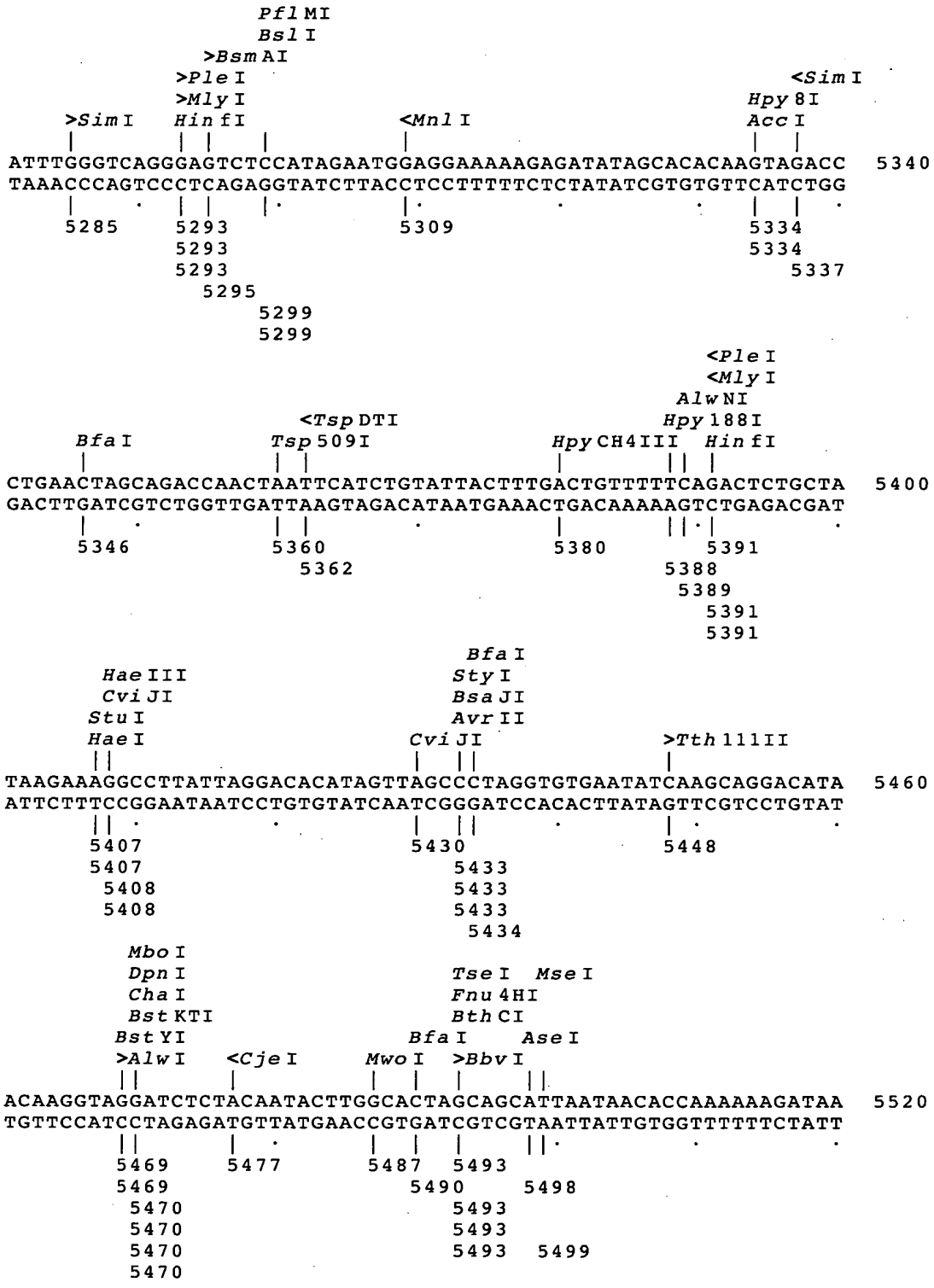
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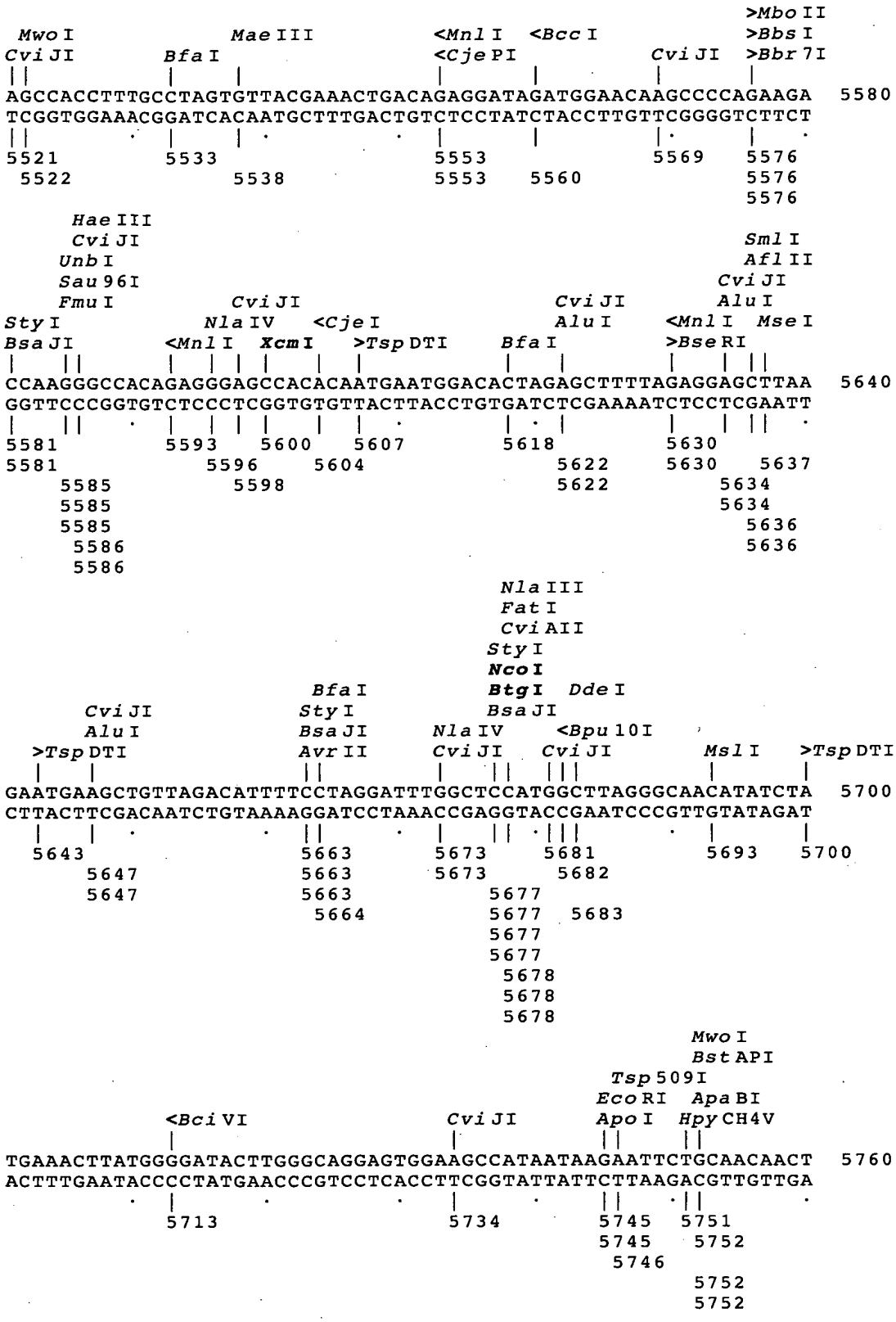
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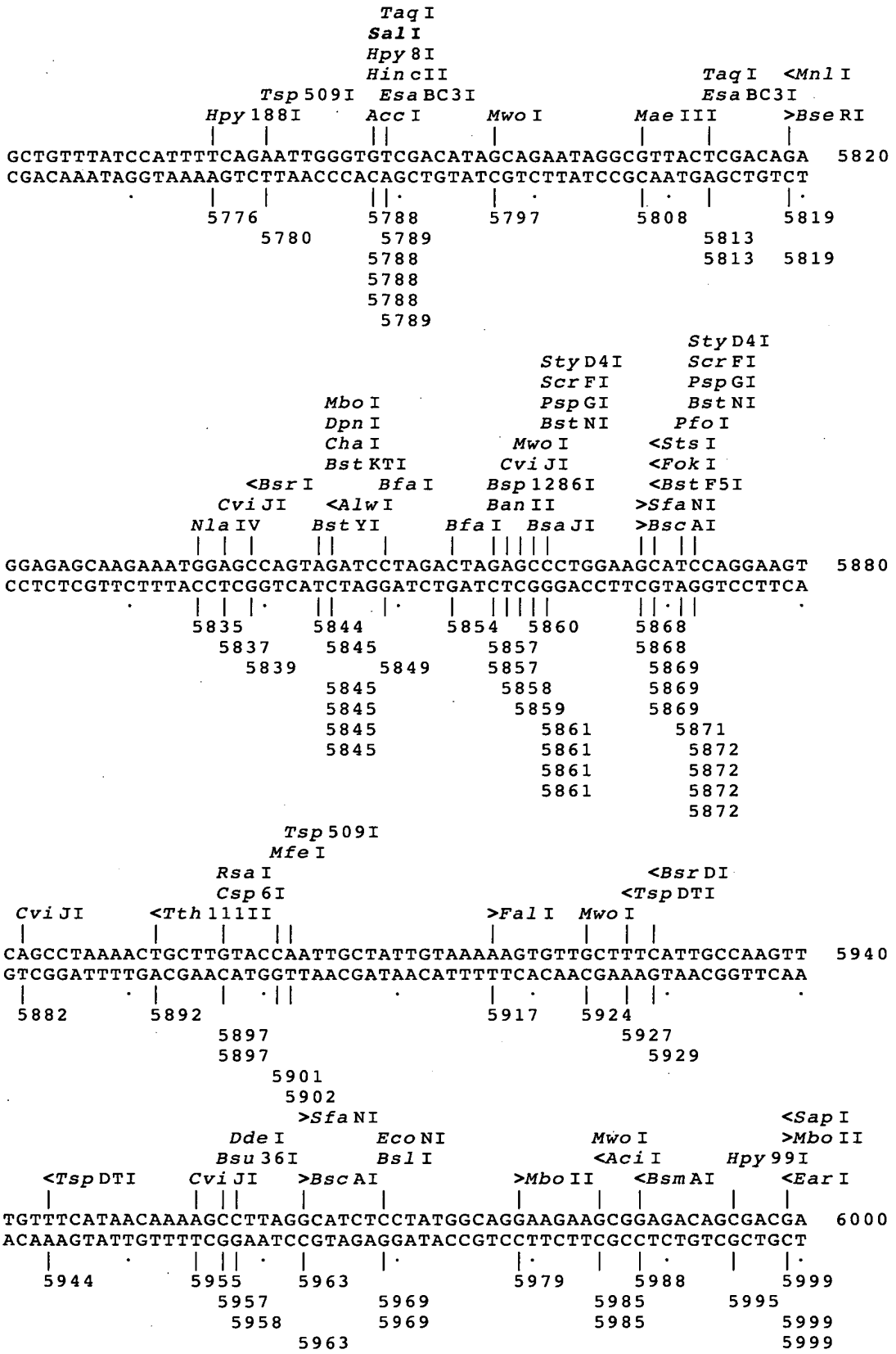
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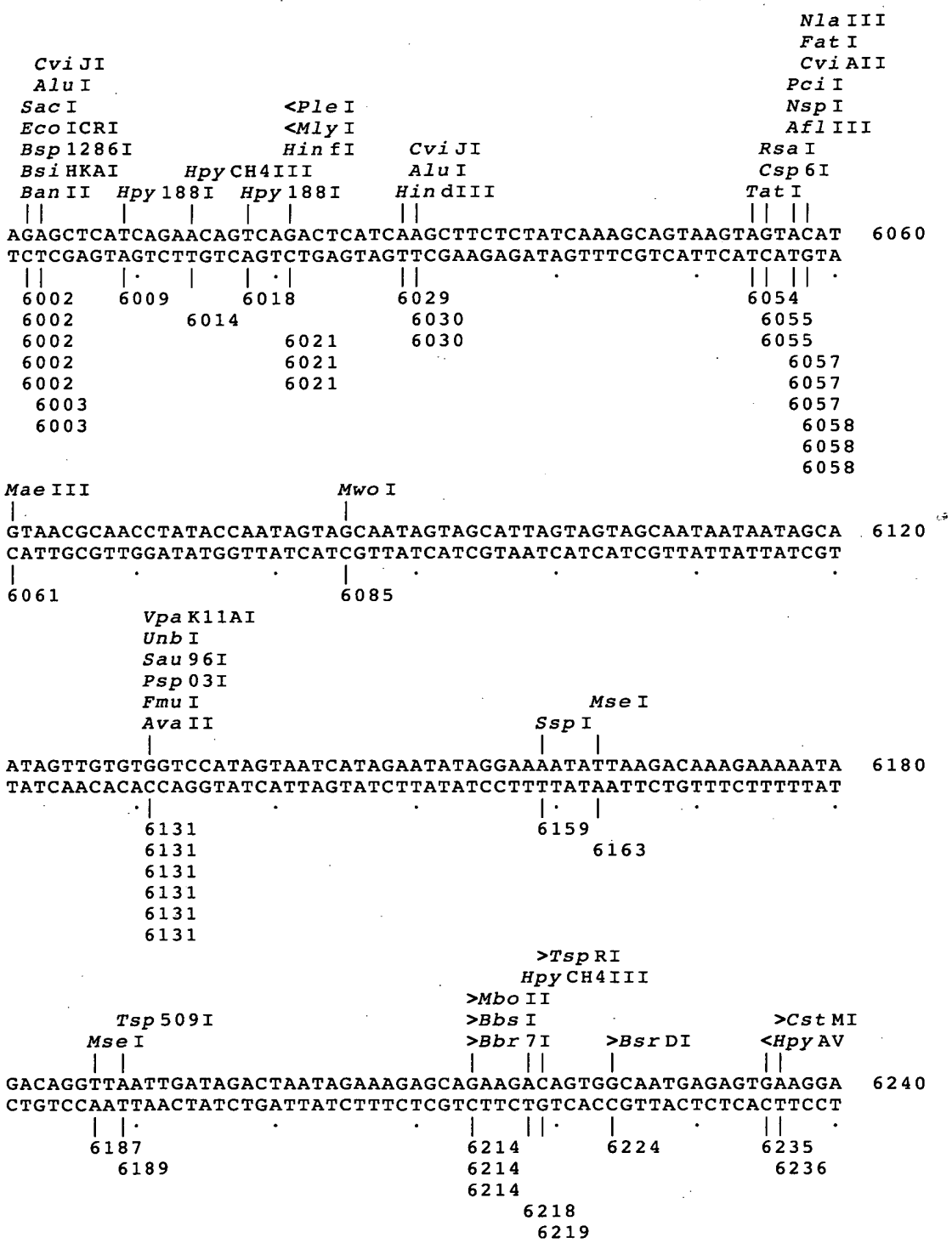
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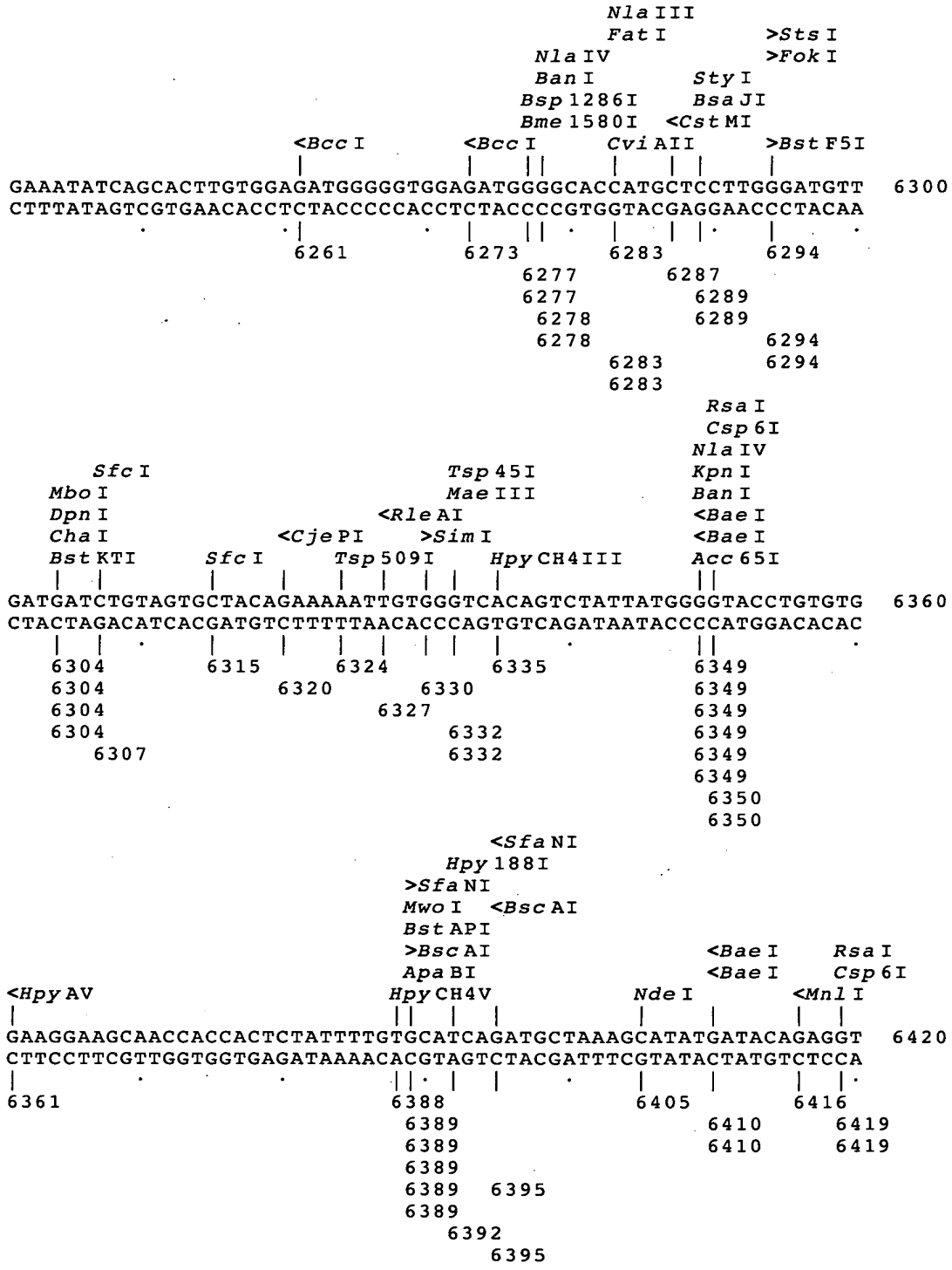
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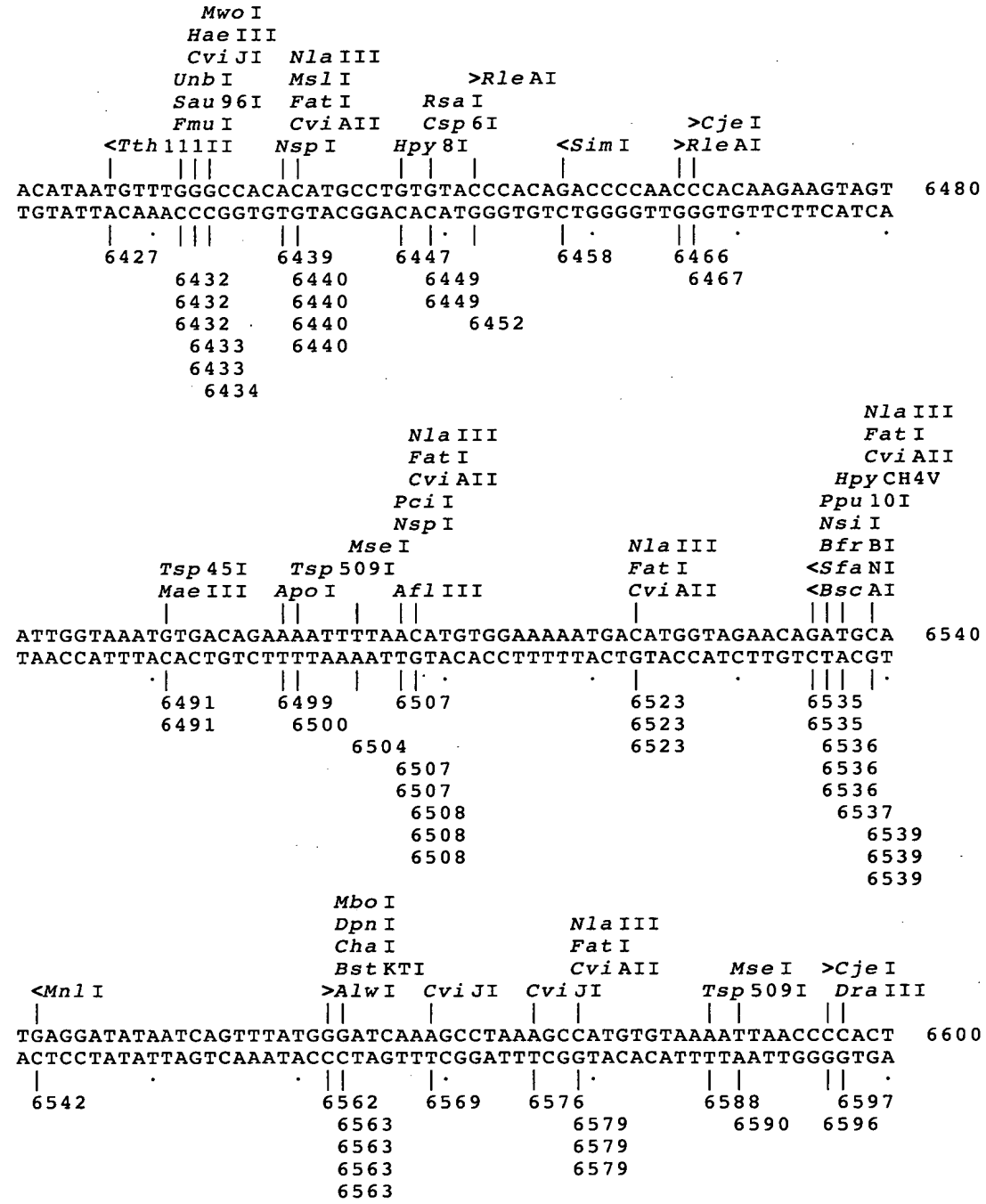
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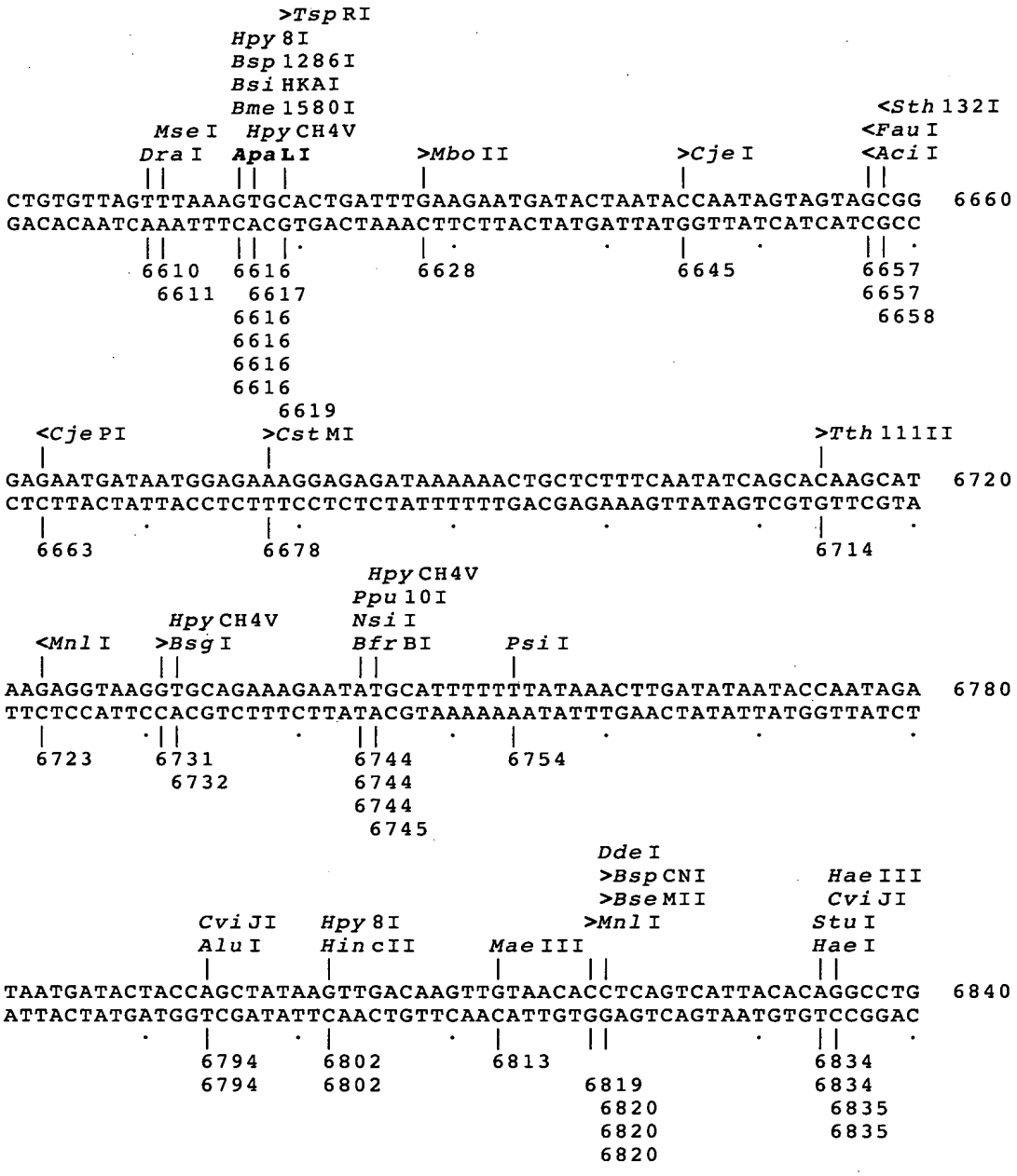
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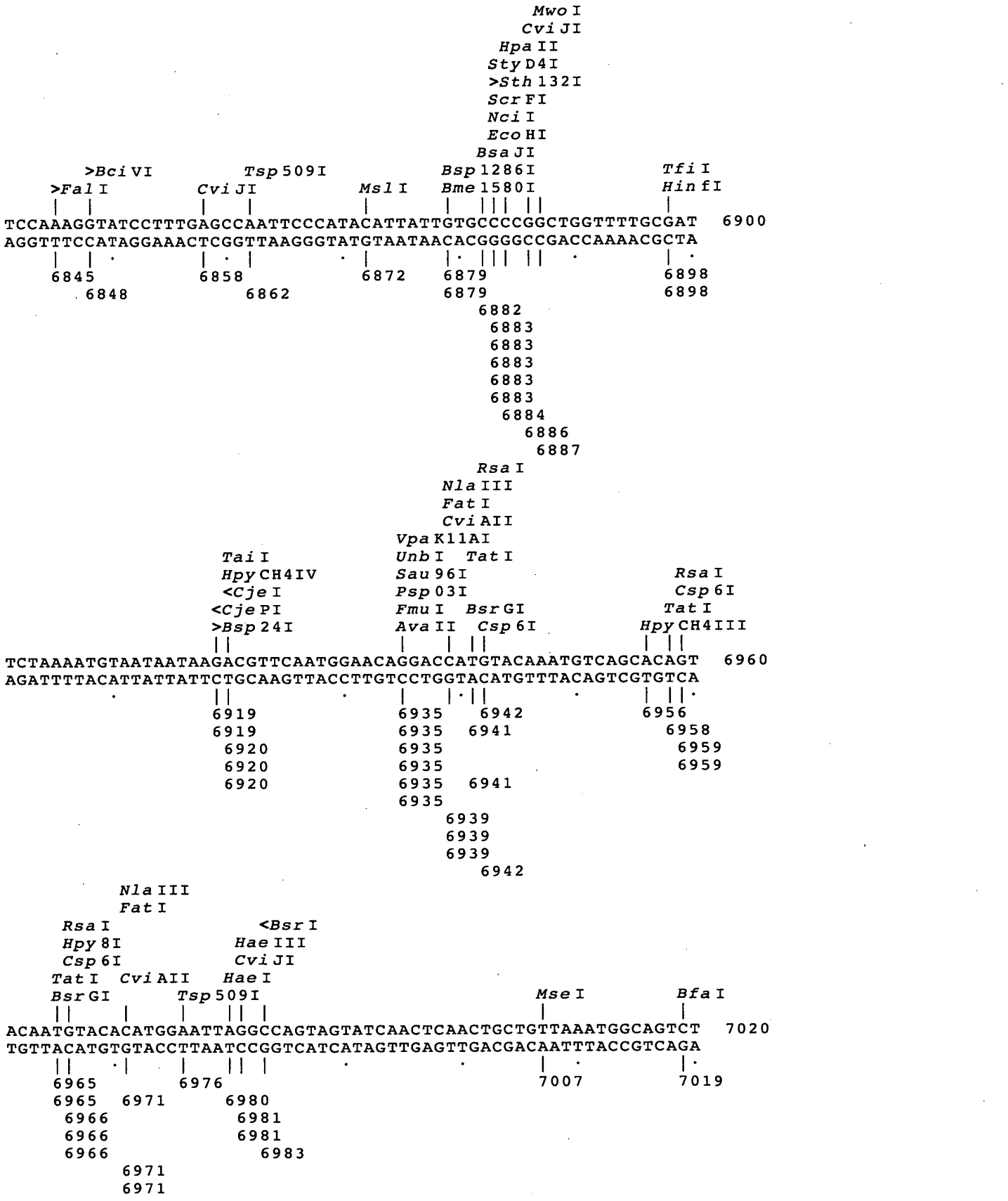
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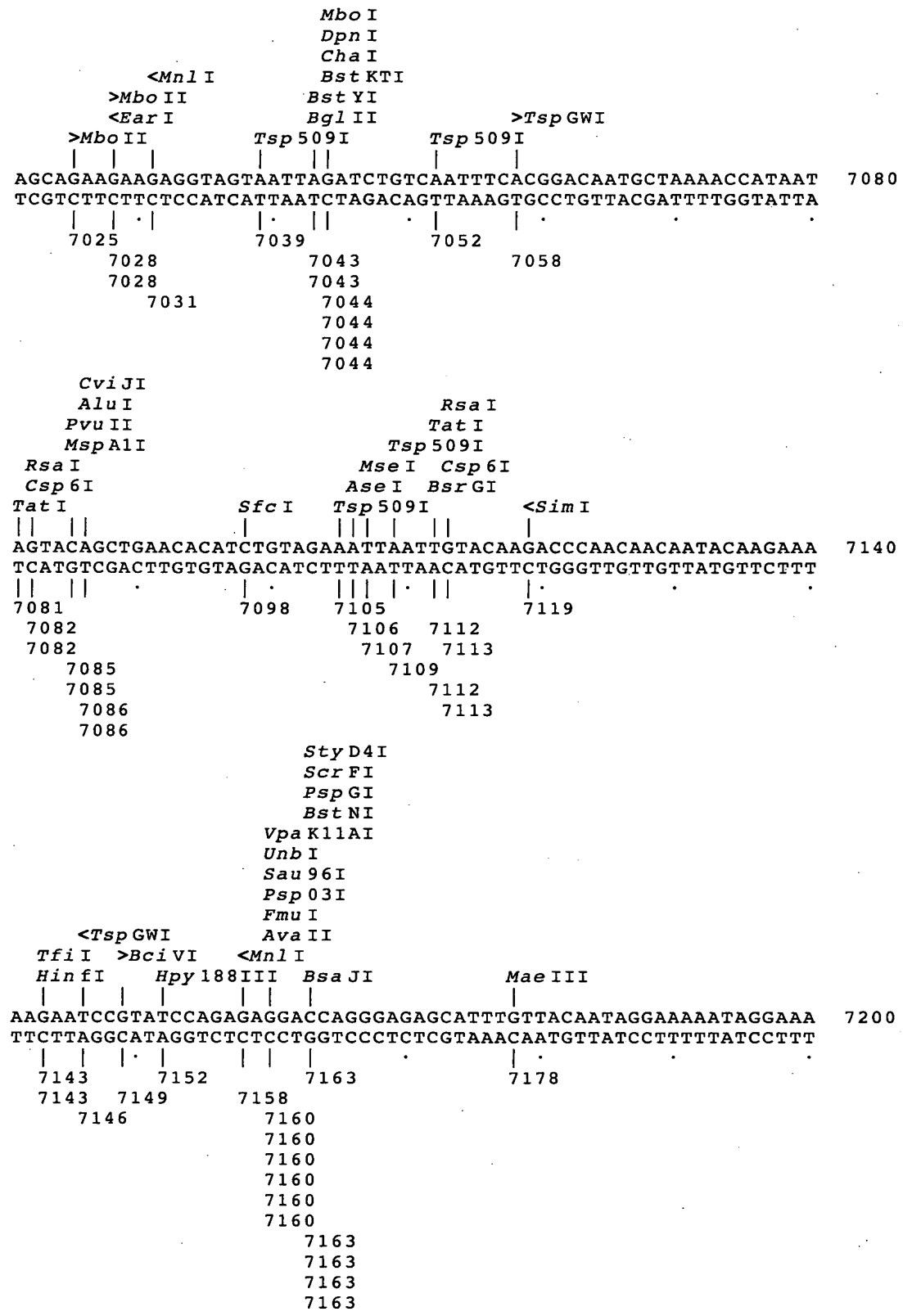
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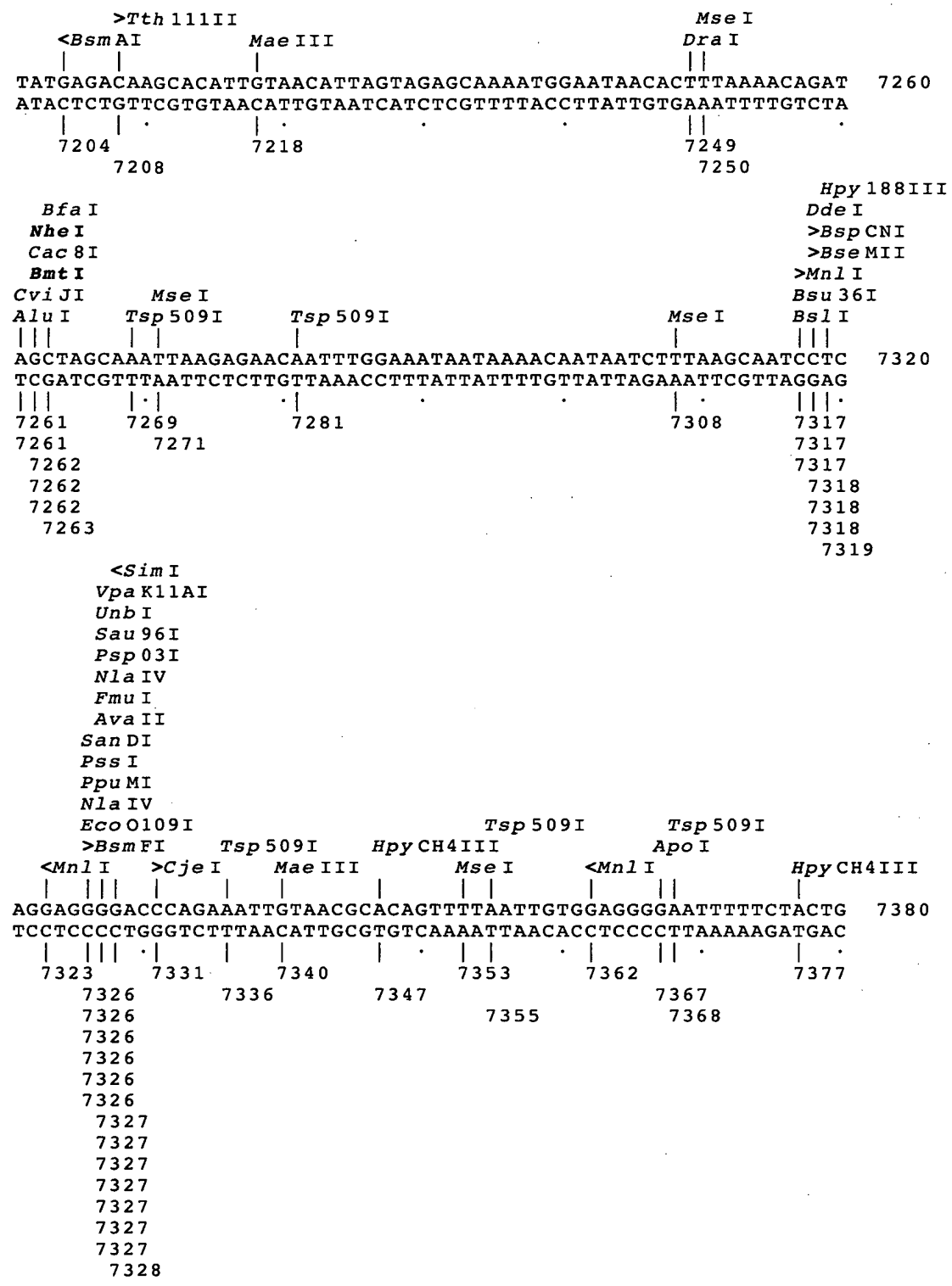
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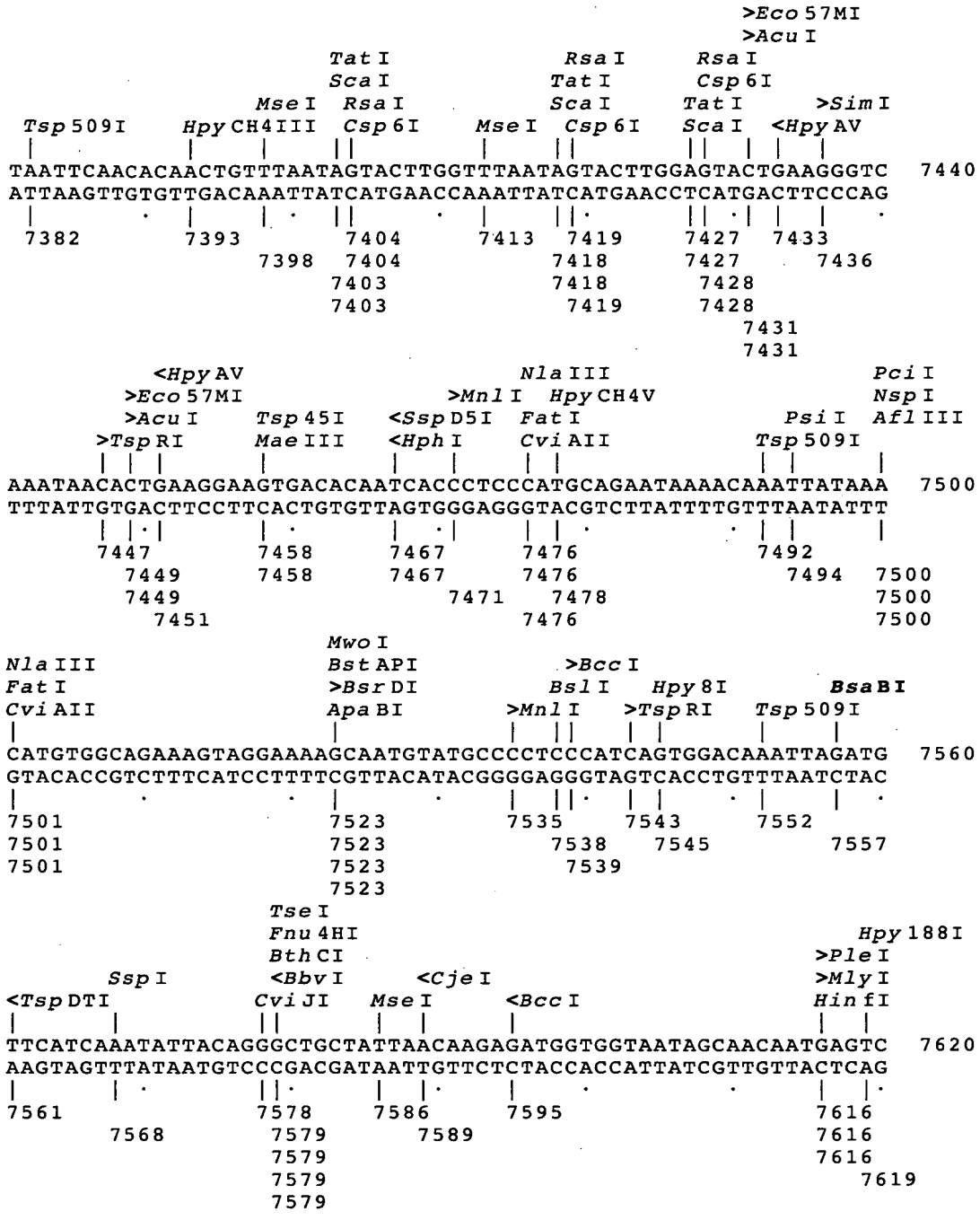
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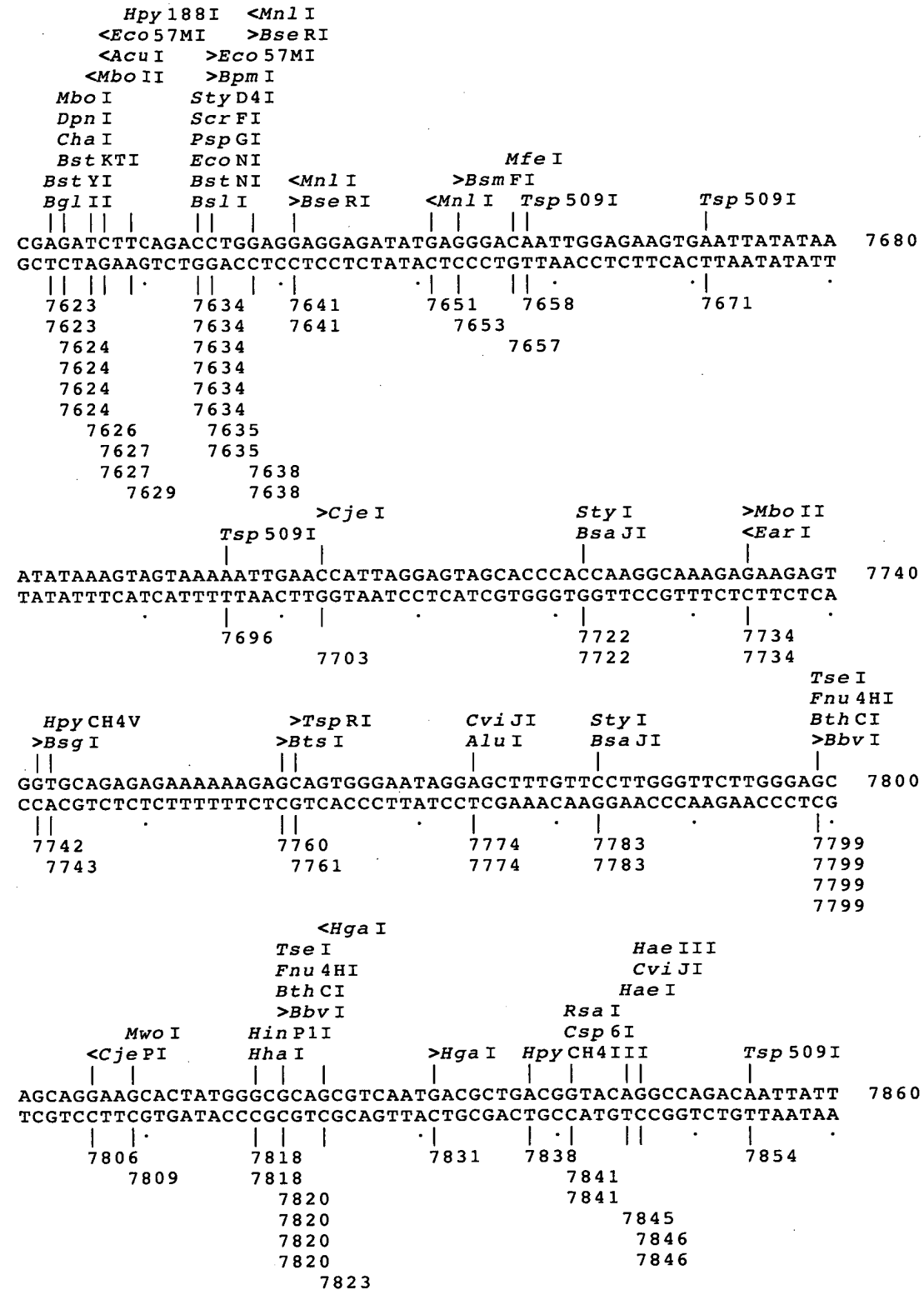
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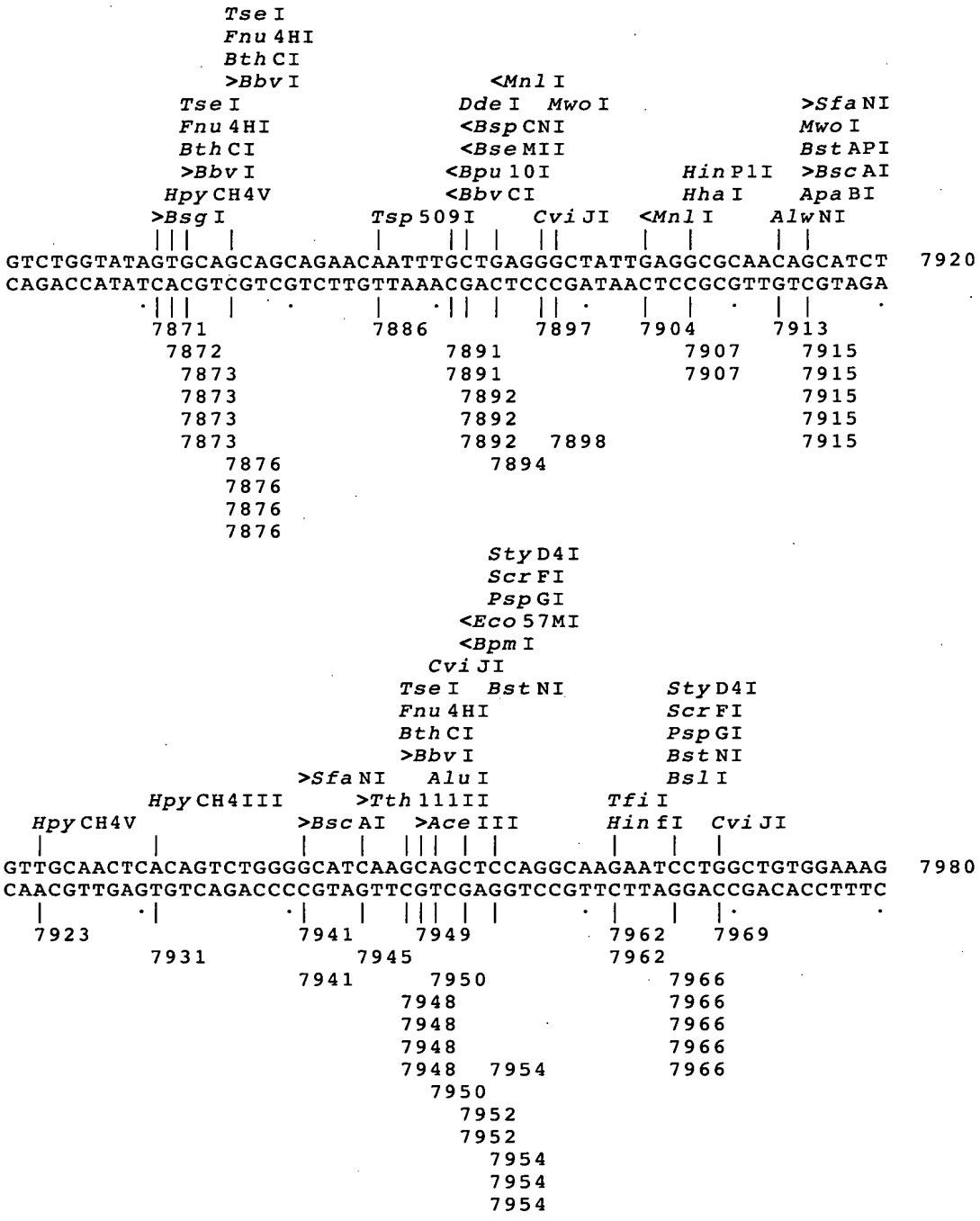
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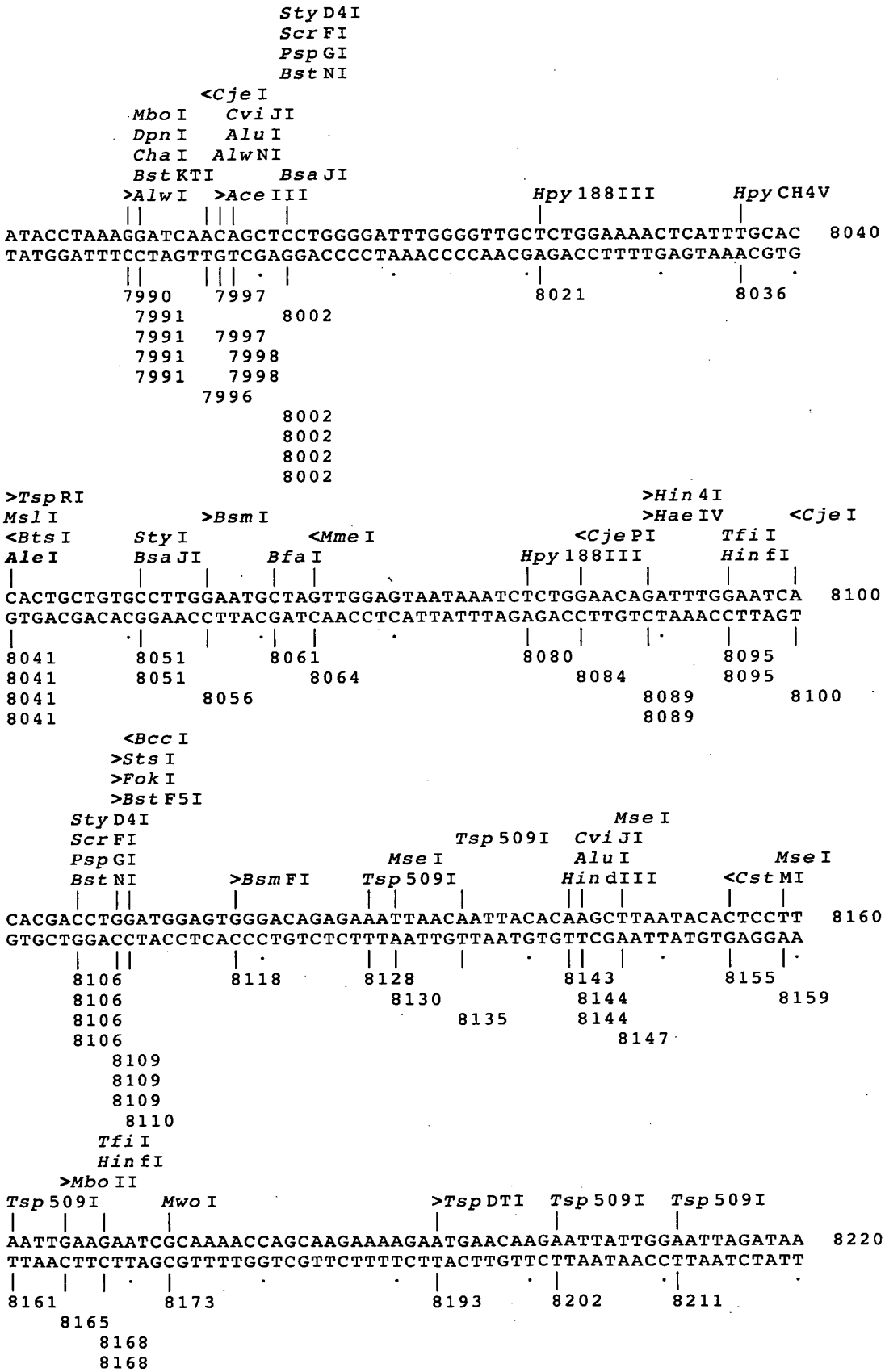
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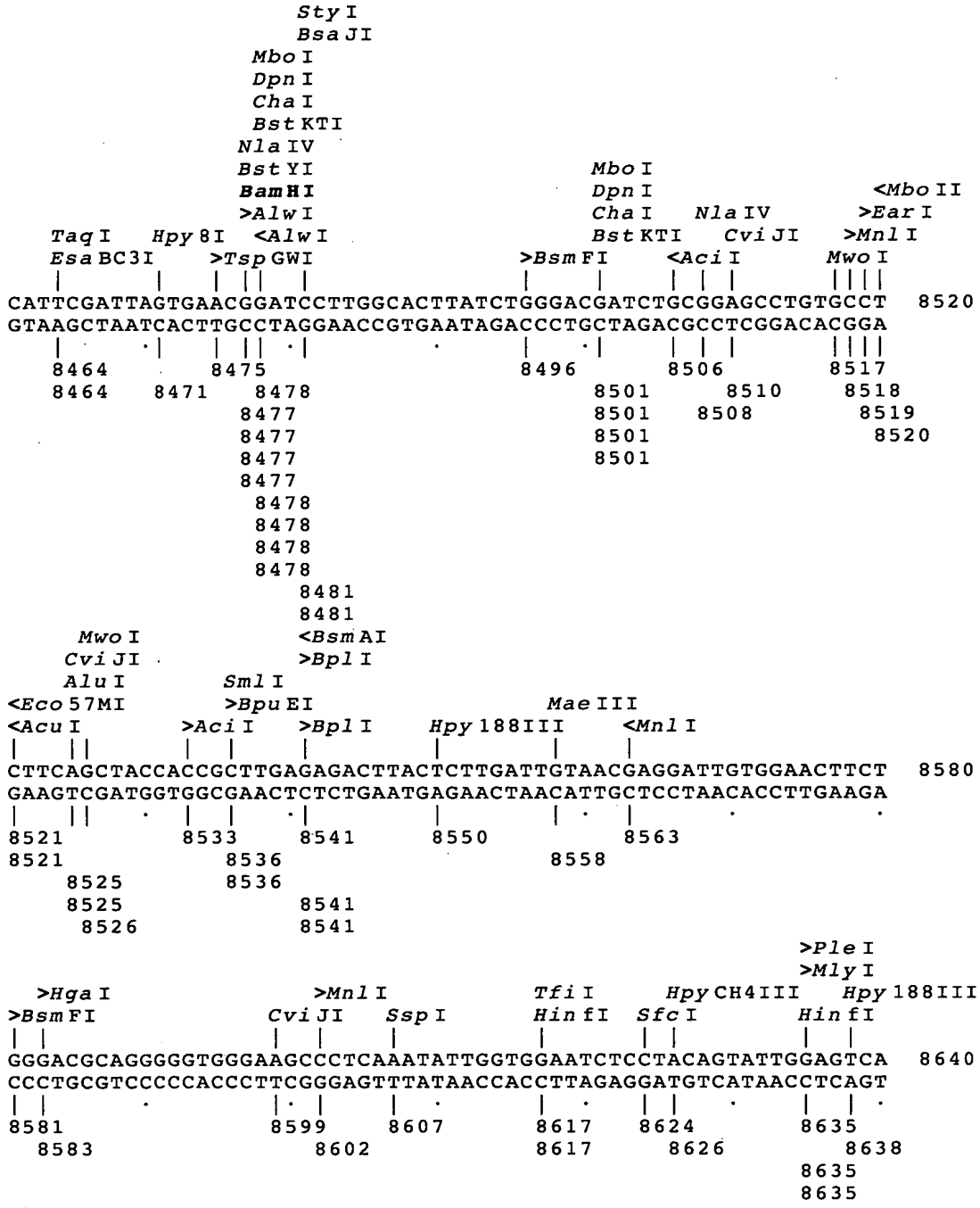
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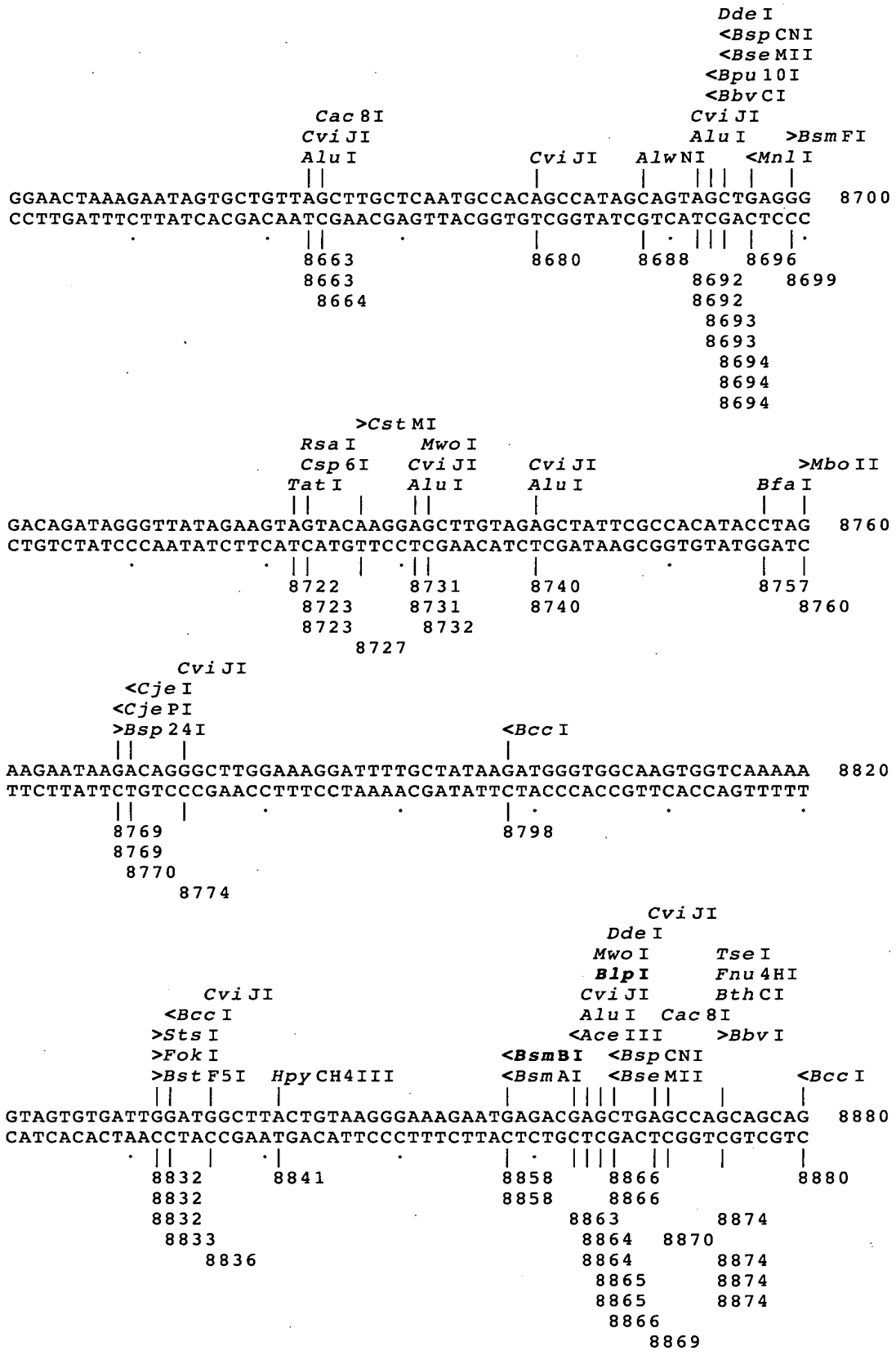
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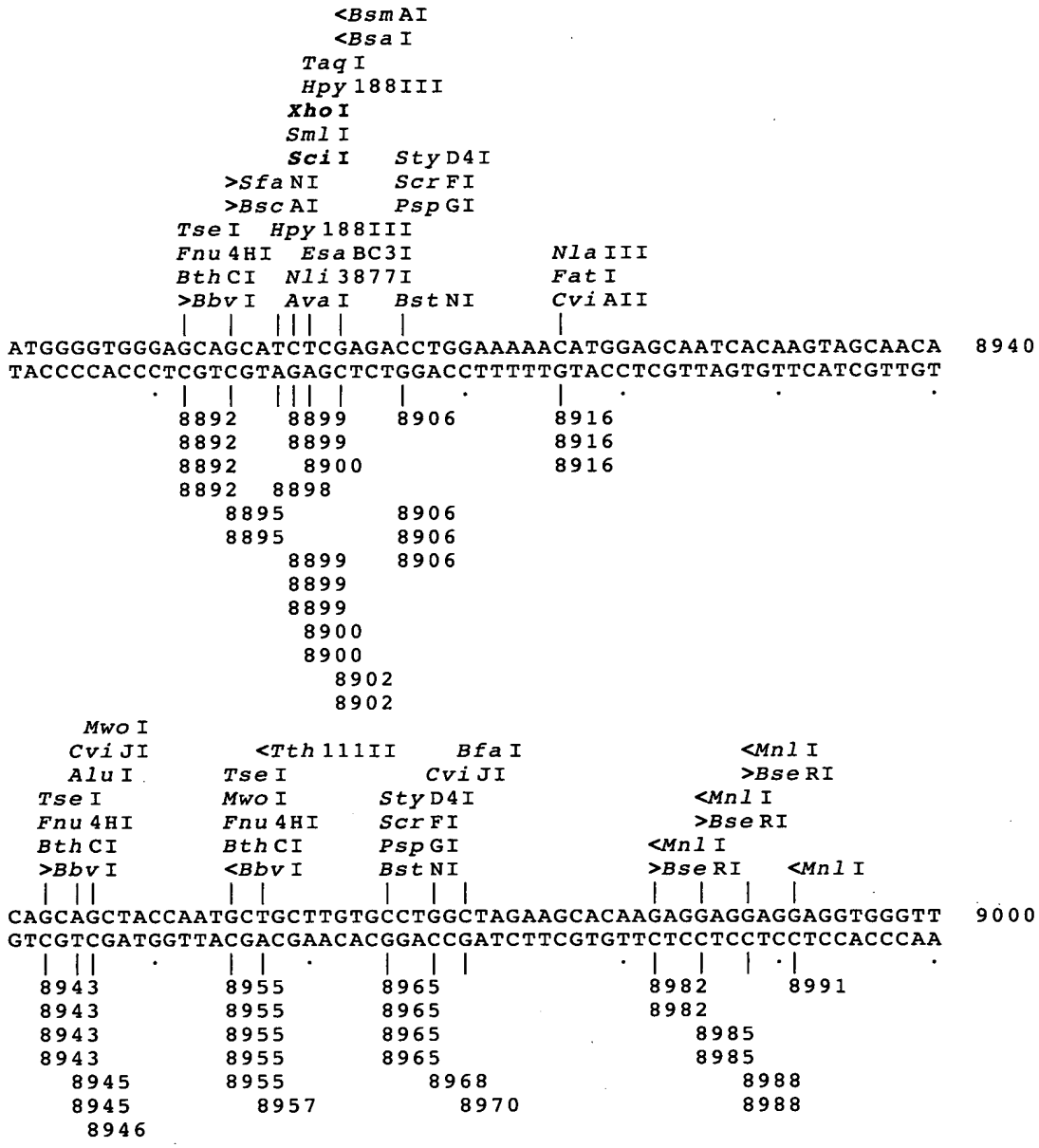
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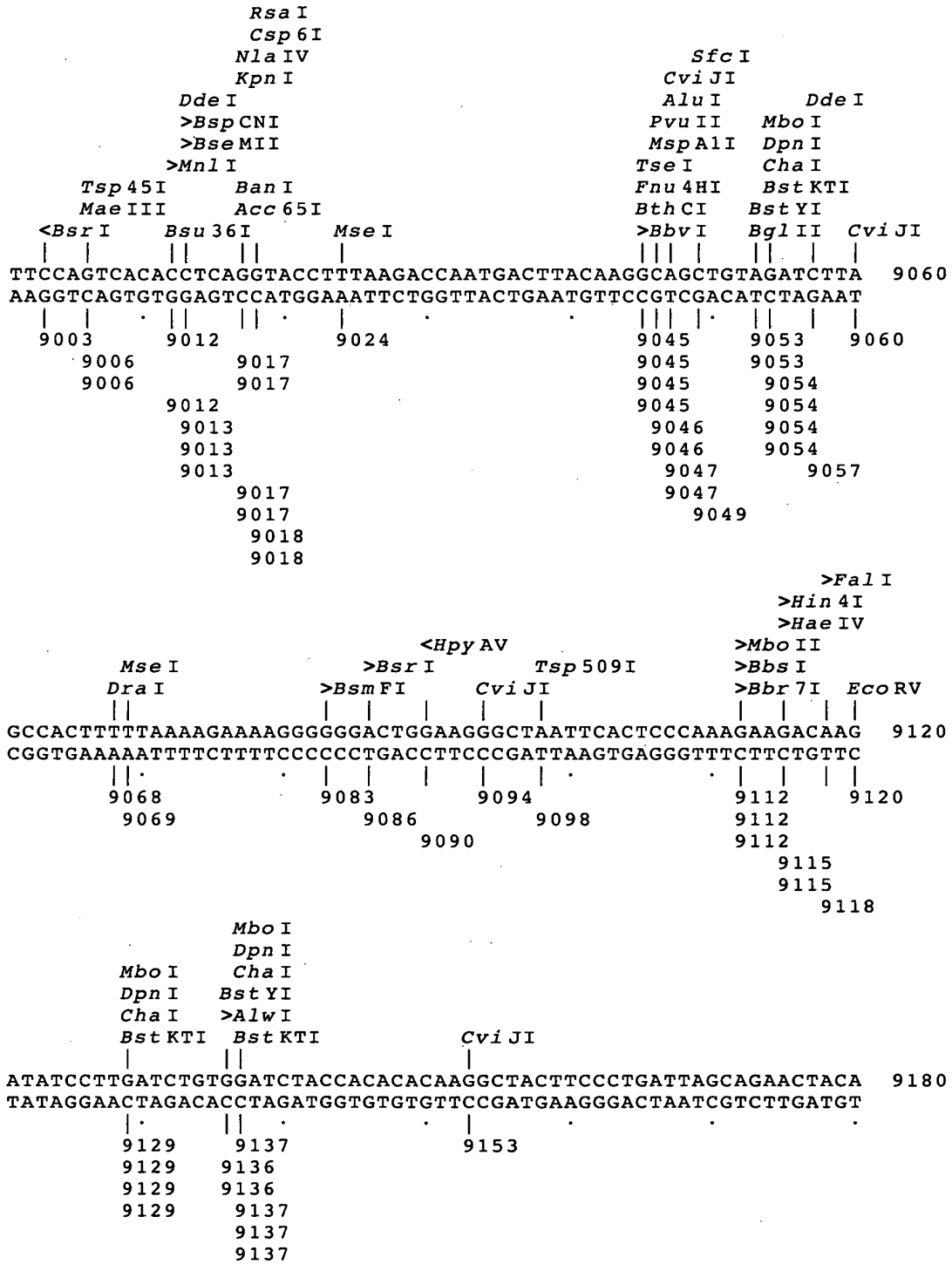
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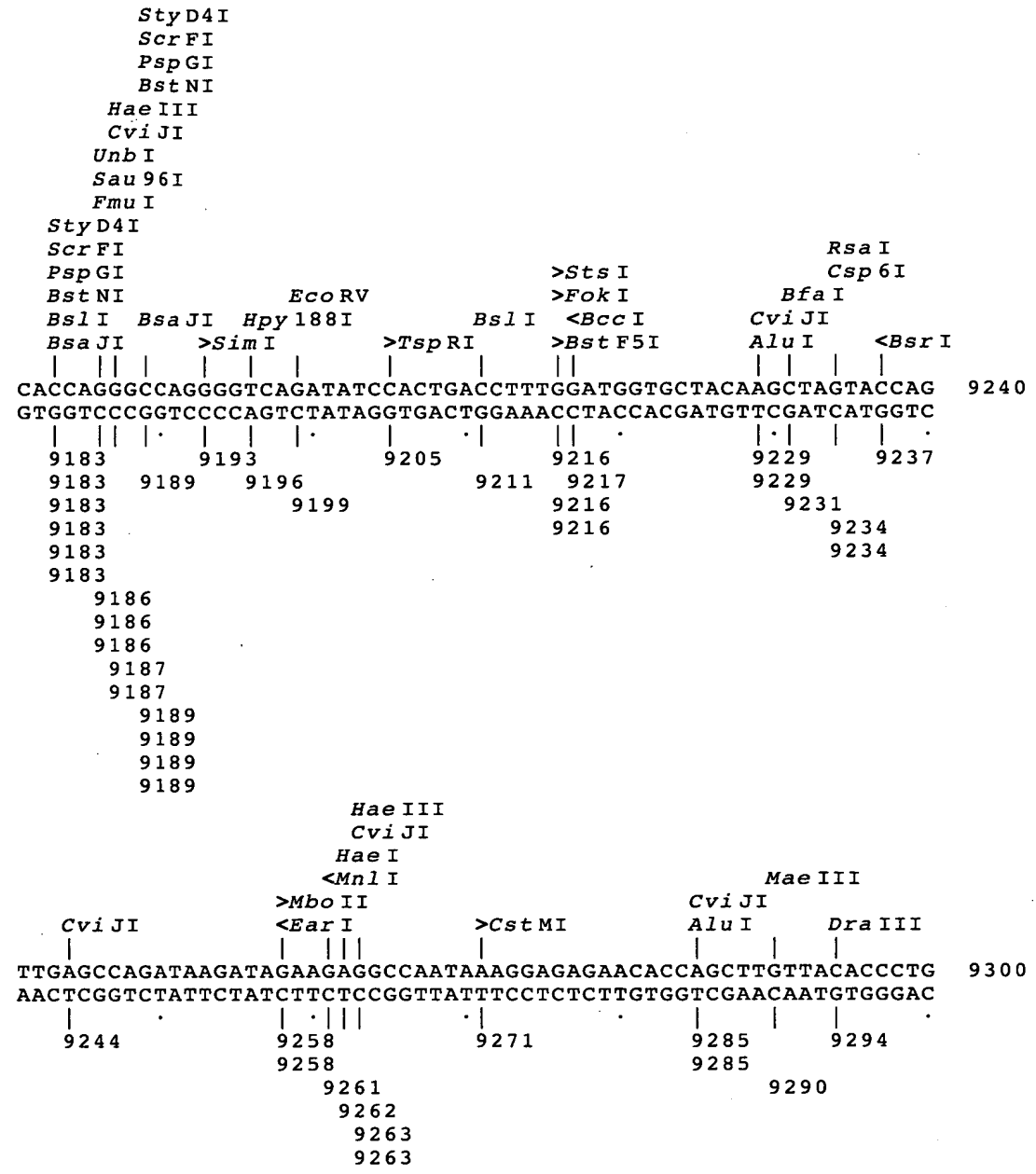
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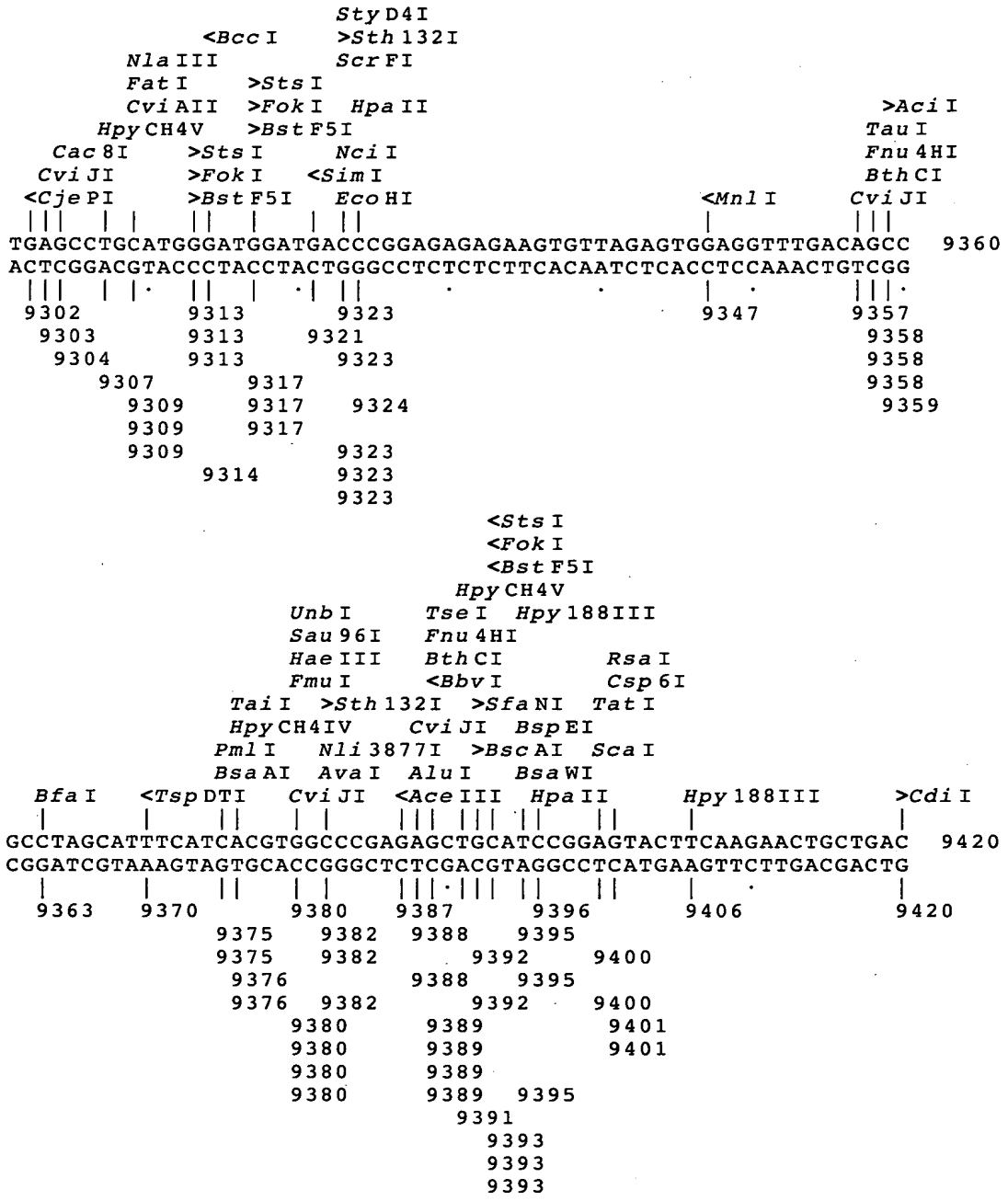
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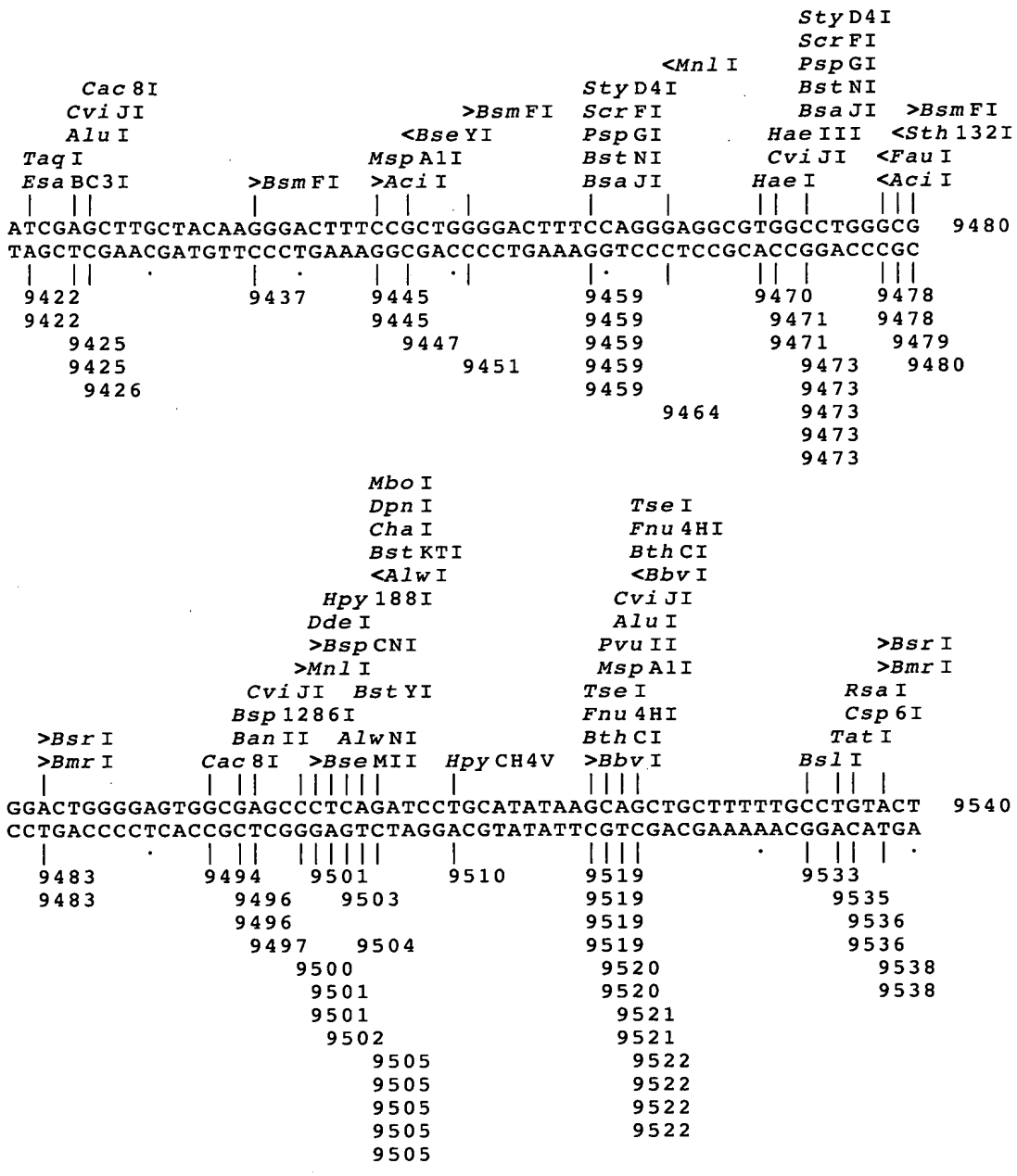
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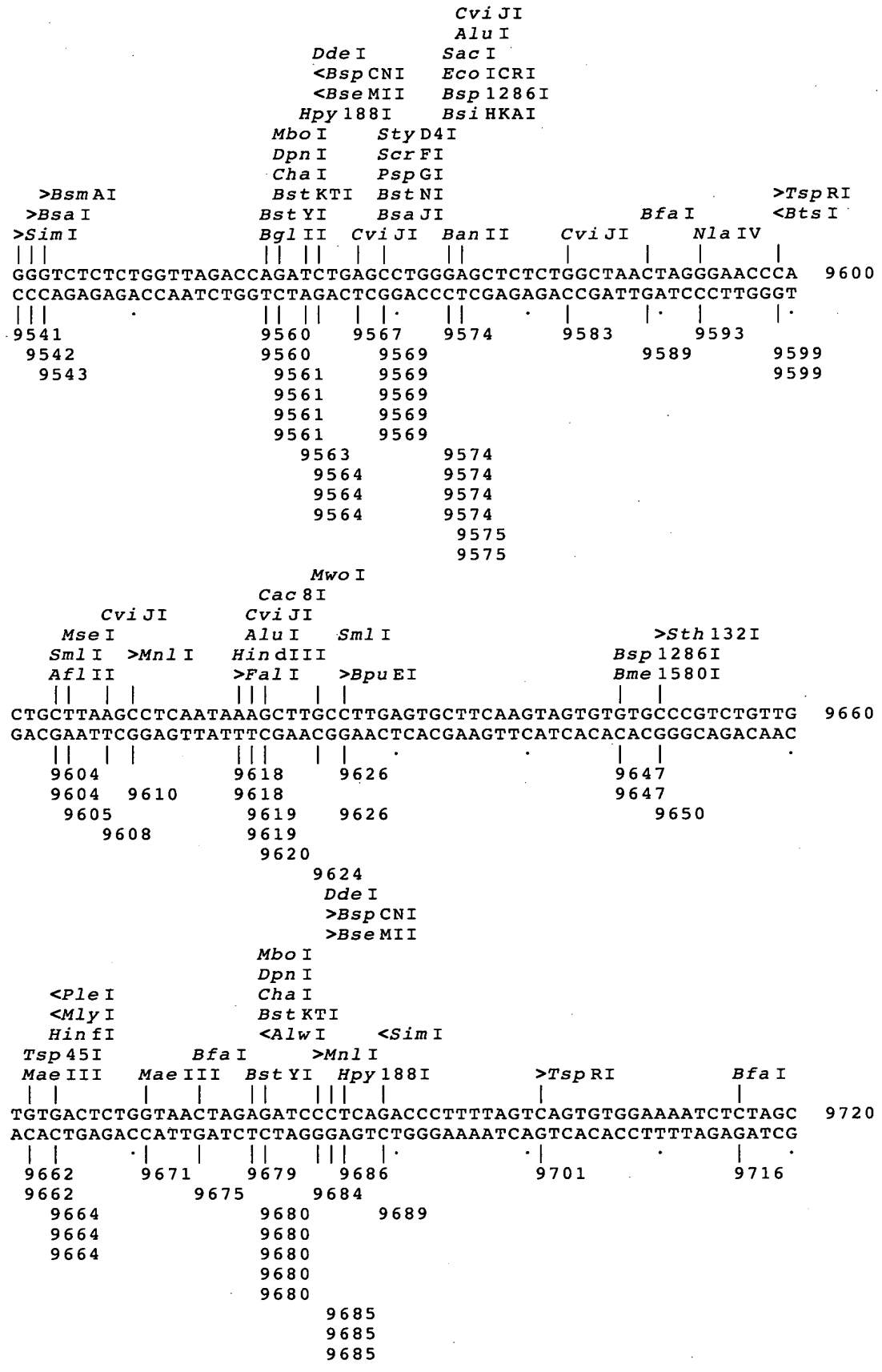
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CCCAGAGAGACCAATCTGGTCTAGACTCGGACCCTCGAGAGACCGATTGATCCCTTGGGT
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HindIII
Bsp1286I
AflII
>FalI
>BpuEI
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DpnI
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ChaI
<MlyI
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MaeIII
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A Single-Stranded Gap in Human Immunodeficiency Virus Unintegrated Linear DNA Defined by a Central Copy of the Polypurine Tract

PIERRE CHARNEAU AND FRANÇOIS CLAVEL*

Unité d'Oncologie Virale, Département SIDA et Rétrovirus, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France

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The structure of unintegrated human immunodeficiency virus type 1 (HIV-1) DNA from acutely infected human lymphoid cells was analyzed by nuclease S1 cleavage. We observed a unique, discrete single-stranded gap in unintegrated linear DNA molecules, located near the center of the genome. Oligonucleotide primer extension experiments determined that the downstream limit of this gap coincides with the last nucleotide of a central copy of the polypurine tract found in all sequenced lentivirus genomes. Other retroviruses have only one copy of the polypurine tract at the 5′ boundary of the 3′ long terminal repeat, which has been shown to determine initiation of retroviral DNA plus-strand synthesis. We conclude from our observations that the central repeat of the polypurine tract can create an additional site for plus-strand synthesis initiation in lentiviruses. The central single-stranded gap was not found in circular DNA molecules, the vast majority of them carrying only one long terminal repeat. This finding suggests that the generation of such circular molecules is associated with early DNA ligation events.

Retroviruses replicate through reverse transcription of their RNA genomes into a double-stranded DNA molecule. Retroviral genes are expressed from an integrated copy of this double-stranded DNA genome, the provirus. Both strands of the retroviral DNA genome are synthesized by the virus-encoded reverse transcriptase, which has both RNA- and DNA-dependent DNA polymerase activities (10, 38, 41). The template for minus-strand synthesis is viral genomic RNA, and the template for plus-strand synthesis is the newly reverse-transcribed minus strand, following removal of RNA from the RNA-DNA hybrid by the RNase H activity associated with reverse transcriptase (8, 11). Synthesis of the minus strand and subsequently of the plus strand is initiated near the 5′ end of the respective template. The corresponding short segments (minus- and plus-strand strong-stop DNAs) are further transferred to the other end of the template, resulting in the formation of the long terminal repeats (LTRs), present at each end of the provirus (10, 30, 37). The primer for minus-strand synthesis is the 3′ end of a tRNA molecule packaged in the viral particle together with genomic viral RNA and hybridized to the primer binding site located at the 3′ boundary of the U5 region (35, 36, 39). The initiation site of the plus strand has been deduced from analysis of reverse transcription reactions products obtained either in vitro or from detergent-disrupted virions and from the sequences of proviral molecular clones. This site is located immediately 3′ of a polypurine tract (PPT) representing the 5′ boundary of the U3 region (21, 22) (Fig. 1). It has been proposed that the PPT is used to define an RNA primer by specific cleavage of the RNA template at this site by the reverse transcriptase-associated RNase H (20, 23, 27, 32). Indeed, in murine retroviruses, in vitro reverse transcription reactions reveal RNA primers that remain associated with the elongating plus strand and are heterogeneous in length (7, 26). Unlike most retroviruses, human immunodeficiency viruses (HIV) and other lentiviruses have two copies of the PPT, one at the border of the 3′ LTR and the other located near the middle of the genome, within the *pol* coding region (5, 12, 33, 43). Previous experiments on visna virus, the prototypic lentivirus, have shown that unintegrated DNA molecules display a single-stranded gap located approximately in the same area (3, 14). Other viral DNA genomes carry single-stranded gaps or nicks. This is the case for hepatitis B virus and cauliflower mosaic virus, in which synthesis of viral DNA genome involves a reverse transcription step.

We have tested here the hypothesis that the HIV central PPT represents an additional initiation site for the synthesis of the plus strand of HIV DNA. We show that HIV linear unintegrated DNA molecules carry a discrete single-stranded gap whose downstream limit is the last nucleotide of the central PPT, indicating that this structure is likely used to initiate plus-strand synthesis at the center of the genome. We also show that the single-stranded gap is absent from circular molecules. Since it is known that such circular molecules are formed only after transport of reverse transcription products into the nucleus, we propose that nuclear ligation and DNA repair events result in both the closing of circular DNA molecules and filling of the gap.

MATERIALS AND METHODS

Cells and viruses. MT4 cells were a gift from M. David Hogan, Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, Md. These cells, which are transformed by human T-cell leukemia virus type I, were shown to allow acute cytopathic HIV-1 infection (13). CEM clone 13 cells (28) were derived from the human lymphoid cell line CEM (ATCC CCL119) and express high levels of CD4 antigen. Cells were maintained in RPMI 1640 medium (GIBCO Laboratories) supplemented with 10% fetal calf serum.

The viral isolate used in our experiments was HIV-1_{bru} (2),

* Corresponding author.

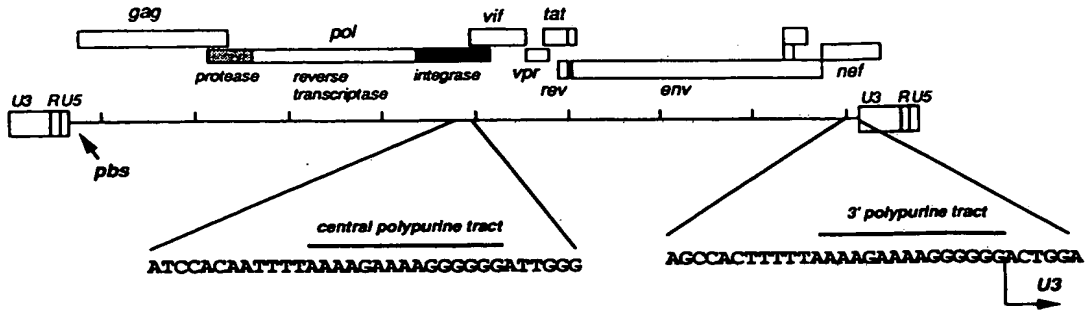

FIG. 1. Positions of the two PPTs on the HIV-1 genome. Shaded areas in the *pol* open reading frame indicate the regions coding for different functions. pbs, Primer binding site, representing the initiation site for minus-strand synthesis.

recovered following transfection of COS cells with infectious proviral molecular clone pBRU-2 (24a). Cells were infected at a multiplicity of 1:10 (1 50% tissue culture infective dose per 10 cells) with virus from a frozen (−80°C) stock produced on MT4 cells and titrating 8×10^5 tissue culture infective doses per ml on MT4 cells. Following infection, cultures were monitored for cytopathic effect, which in the described conditions of infection appeared approximately 3 days after infection. At that time, cells were harvested for DNA isolation.

Analysis of viral DNA. Low-molecular-weight DNA was extracted from infected cells by Hirt extraction (15). Nuclease S1 (Appligene, Strasbourg, France) was used at 1.5 U/μg of DNA after addition of 1:10 volume of 10× S1 buffer (300 mM sodium acetate [pH 4.6], 500 mM NaCl, 10 mM $ZnCl_2$) and incubated at 37°C. For double digestions with a restriction enzyme and nuclease S1, DNA (10 μg) was first digested with the restriction enzyme; then 10× nuclease S1 buffer and nuclease S1 (15 U) were added to the reaction mixture, and the sample was further incubated at 37°C. DNA was then subjected to electrophoresis on 1% agarose gels that did not contain ethidium bromide and analyzed by Southern blotting (34).

The nucleotide position numbers used here to describe DNA fragments start at the first nucleotide of a linear HIV DNA genome (5′ end of the U3 region in the 5′ LTR).

Two probes were used in hybridization experiments. The 5′ probe was a *PstI* fragment spanning the *gag* region from positions 1415 to 2839, and the 3′ probe was a *KpnI* fragment spanning the *env* region from positions 6343 to 9005. Both probes were obtained from HIV-1 molecular clone pNL4-3 (1) and labeled by the random hexamer method (6).

Primer extension. A modification of the primer extension technique was used, based on *Taq* DNA polymerase, which allows multiple cycles of denaturation, annealing, and polymerization. The reaction mix contained 10 μg of Hirt DNA, 0.2 mM each deoxynucleotide, 1.25 U of *Taq* DNA polymerase (Perkin-Elmer Cetus N801-0045), 10 pmol of 5′-end-labeled primer, and 0.5 μl of PMPE reagent (Stratagene) in 10 mM Tris-HCl (pH 8.3)–50 mM KCl–2 mM $MgCl_2$–0.01% gelatin, for a total reaction volume of 50 μl. The first cycle included 30 s for denaturation at 92°C, 1 min for annealing at 50°C, and 1 min for polymerization at 72°C. The following 59 cycles included 10 s at 92°C, 1 min at 50°C, and 1 min at 72°C. As only one primer was included in the reaction, this multicycle primer extension method is distinct from a polymerase chain reaction and resulted in linear, not exponential, amplification of the reaction product.

Oligonucleotide primers were 5′ end labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP to a specific activity of 5×10^5 to 10^6 cpm/10 pmol. Primer pol-1 (5′-ACA ATC ATC ACC TGC CAT CTG) anneals to the plus strand at position 5085. Primer pol-2 (5′-TCC AAA GTG GAT CTC TGC TGT) anneals to the plus strand at position 4951.

The same oligonucleotide primers were used to generate a sequence ladder from a single-stranded M13 template carrying the HIV-1_{bru} plus strand from positions 4688 to 5129. Sequencing was done by the method of Sanger et al. (29). Primer extension and sequence reactions were analyzed in parallel on a 6% polyacrylamide–8 M urea sequencing gel.

RESULTS

A single-stranded gap in HIV-1 unintegrated DNA. We hypothesized that the existence of an additional plus-strand initiation site in the HIV-1 genome could be revealed by the presence of a single-stranded region in HIV-1 unintegrated DNA molecules. To examine this possibility, low-molecular-weight DNA was selectively extracted (15) from acutely HIV-1-infected MT4 cells (13), treated with nuclease S1, and analyzed by Southern blotting with a probe spanning most of the 3′ half of the HIV-1 genome. Figure 2 shows the kinetics of nuclease S1 digestion of HIV-1 unintegrated DNA. In undigested DNA, three bands were observed. The position of the middle band, around 9.5 kb, is consistent with linear full-length molecules, and the position of the lower band, around 6 kb, is consistent with closed (supercoiled) circles. The upper band, which has an apparent size of approximately 15 kb, is likely to be open (relaxed) circular molecules. After 5 min of digestion with nuclease S1 (15 U/10 μg of DNA), major changes occurred. The band corresponding to linear molecules turned into a doublet; the upper band had the size of intact linear molecules (9.5 kb), and the lower one, at approximately 9 kb, was consistent with the size of linearized circles with one LTR. Simultaneously, the closed-circles 6-kb band completely disappeared, while the intensity of the open-circles band increased slightly. More strikingly, a new 5-kb band appeared, corresponding to the 3′ half of linear molecules cut into two fragments by nuclease S1. The other fragment, of similar size, could be detected in a distinct hybridization experiment with a 5′ probe (data not shown). This finding indicates the presence of a unique nuclease S1-sensitive site in HIV unintegrated DNA molecules located approximately at the center of the genome.

The complete disappearance of the closed circles, even

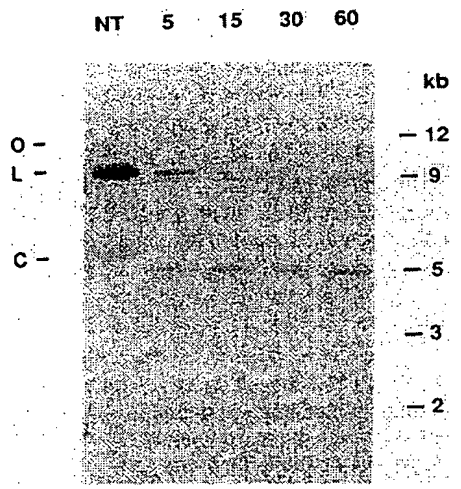

FIG. 2. Kinetics of nuclease S1 action on HIV-1 unintegrated DNA. Shown is a Southern blot of low-molecular-weight DNA from HIV-1-infected MT4 cells, hybridized to a probe representing most of the 3′ half of the HIV-1 genome (see Fig. 3B). Lanes: NT, no nuclease S1 treatment; 5, 15, 30, and 60, treatment with S1 nuclease (1.5 U/μg of DNA) for 5, 15, 30, and 60 min, respectively. O, Open circles, L, linear molecules; C, closed circles.

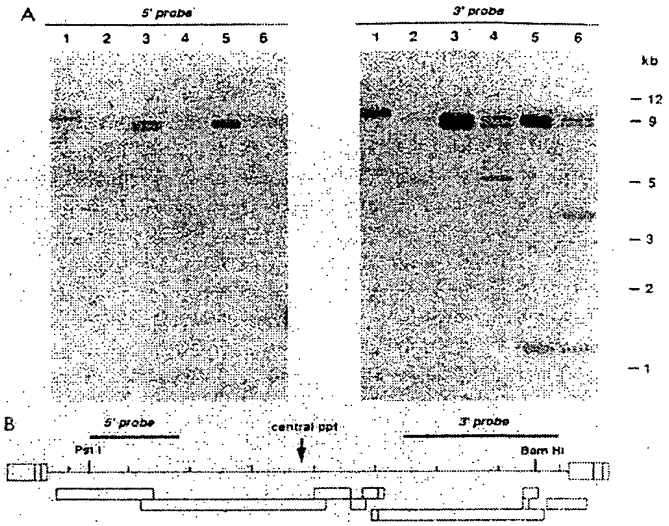

FIG. 3. Position of the single-stranded gap on the HIV-1 genome. (A) Two autoradiograms from the same Southern blot, hybridized to two different probes as indicated at the top. Low-molecular-weight DNA from HIV-1-infected MT4 cells was analyzed by nuclease S1 treatment either with no previous restriction enzyme digestion (lanes 1 and 2), after digestion with *Pst*I (lanes 3 and 4), or after digestion with *Bam*HI (lanes 5 and 6). Lanes 1, 3, and 5, No nuclease S1 treatment; lanes 2, 4, and 6, treatment with nuclease S1 (1.5 U/μg of DNA). (B) Positions of the corresponding probes on the HIV-1 genome, relative to the central PPT and to the unique *Pst*I and *Bam*HI restriction sites.

after 5 min of nuclease S1 treatment, can be best explained by a previously described high sensitivity of supercoiled circular DNA molecules to nuclease S1 (19). Therefore, we assumed that at this stage of incomplete nuclease S1 digestion, the closed circles were cleaved at random sites, into open circles if only one strand was cut (this explains the increase in open circles) and into linear molecules when S1 nuclease further cleaved at the resulting nicks.

With further digestion, the open-circles band gradually disappeared, together with the full-length linear molecules, while the band corresponding to linearized one-LTR circles remained unaffected. The gradual disappearance of open circles was due to their cleavage at random nicks that relate to their relaxed physical state: S1 cleavage at those sites linearized them. Meanwhile, linear molecules were further cleaved at the central S1-sensitive site, generating more of the probe-reactive 5-kb fragment: after 1 h of treatment, very little of the full-length linear molecules remained.

The central single-stranded gap is discrete and unique. We further attempted to localize and define the structure of the HIV-1 central nuclease S1-sensitive site. Figure 3A shows a Southern blot analysis of nuclease S1 cleavage products of HIV-1 unintegrated DNA, obtained from acutely HIV-1-infected MT4 cells, combined with digestion by two different restriction enzymes. In these experiments, complete restriction enzyme digestions were performed, followed by partial nuclease S1 digestion (20 min with 15 U/10 μg of DNA). The digestion products were examined by hybridization to two different HIV-1 probes: a 5′ probe, in the *gag* region, spanning from nucleotide positions 1415 (from the start of U3) to 2839, and a 3′ probe, spanning most of the *env* and *nef* regions, from nucleotide positions 6343 to 9005.

In the HIV-1_{bru} genome, *Pst*I has a unique site in the *gag* coding region at position 1415. Digestion of HIV-1 unintegrated DNA with *Pst*I (Fig. 3A, lanes 3) cut the linear molecules in two fragments of 8.3 and 1.4 kb, the latter being undetected by either of the two probes used in this experiment. *Pst*I also linearizes circles into a 9-kb product. Nuclease S1 treatment of *Pst*I digestion products (lanes 4) yielded a 5-kb fragment reactive with the 3′ probe and a 3.3-kb fragment reactive with the 5′ probe. This result establishes that both boundaries of the central single-stranded gap are discrete.

After digestion with *Bam*HI (Fig. 3A, lanes 5), which has a unique site in the *env* region at position 8520, the linear DNA molecules were cleaved into two fragments of approximately 8.5 and 1.2 kb, both reactive with the 3′ probe. Treatment with nuclease S1 (lanes 6) released a 3.7-kb fragment detected by the 3′ probe and a 4.9-kb fragment reactive with the 5′ probe. The 1.2-kb fragment, which includes the 3′ PPT, remained unaffected by nuclease S1 treatment. When no restriction enzyme digestion was performed before nuclease S1 treatment, S1 cleavage (lanes 2) released a 5′ 4.9-kb fragment and a 3′ fragment of similar size. Overall, the results from these single- and double-digestion experiments were consistent and could locate the single-stranded gap around position 4900, within 0.2 kb of the central PPT repeat found in the HIV-1_{bru} sequence.

These experiments show that the central single-stranded gap is unique in the HIV-1 genome. Indeed, the sizes of the fragments released by both the single and double digestions excluded the presence of another gap.

The 3′ boundary of the gap is the central copy of the PPT. To locate the 3′ boundary of the single-stranded gap more precisely relative to the central PPT found in all lentiviral genomes, primer extension experiments were conducted, using low-molecular-weight DNA from acutely HIV-1-infected lymphoid cells as a template. We used oligonucleotide primers complementary to the viral plus strand, located

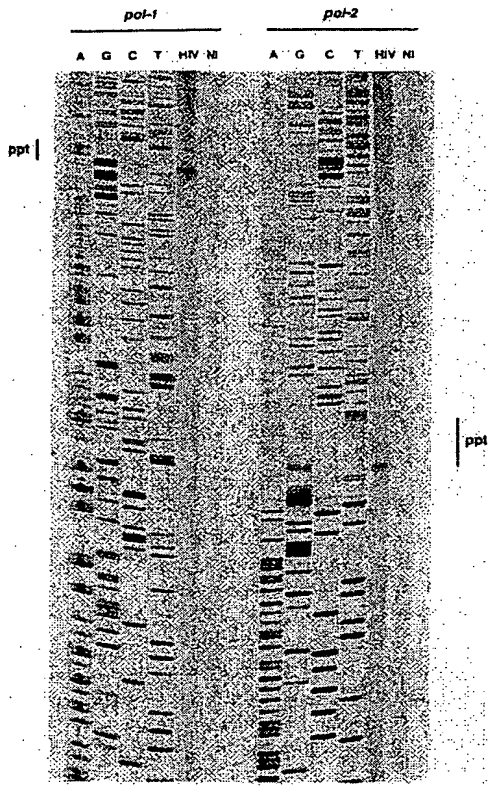

FIG. 4. Localization of the downstream limit of the single-stranded gap by oligonucleotide primer extension. Two primer extension reactions with two different oligonucleotide primers are presented. Primer pol-1 anneals to the HIV-1 plus strand at positions 5085 to 5105, and primer pol-2 anneals at positions 4951 to 4971. Both oligonucleotides were ^{32}P 5′ end labeled and allowed to prime an extension reaction, using low-molecular-weight DNA from HIV-1-infected cells (lanes HIV) or noninfected cells (lanes NI) as templates as described in Materials and Methods. Lanes A, G, C, and T are sequence reactions from a single-stranded M13 template carrying the HIV-1_{bru} plus strand from positions 4688 to 5129, using the indicated oligonucleotide as a primer. ppt, Central copy of the PPT.

downstream to the central copy of the PPT, expecting elongation of these primers to stop precisely at any interruption of the plus strand. To increase the sensitivity, the enzyme used was *Taq*I polymerase, which allowed us to heat denature extension reaction mixes and to carry several cycles of extension for each reaction. Two different antisense 5′-end-labeled oligonucleotide primers were used. Primer pol-2 anneals to the plus-strand 135 nucleotides downstream of the PPT, and primer pol-1 anneals 134 nucleotides further downstream. In parallel with extension reactions, DNA sequence reactions were conducted, using the same oligonucleotide primers on single-stranded M13–HIV-1_{bru} templates, enabling us to precisely locate the stop in primer extension on the HIV-1_{bru} genome (Fig. 4). With both primers we observed a single, discrete stop in primer extension that coincided with the last nucleotide of the central PPT. No equivalent stop was found with low-molecular-weight DNA from uninfected cells. In addition, no HIV-specific signal could be observed with a primer complementary to the minus strand, located upstream of the central PPT (data not shown). This result demonstrates that the 3′ boundary of the HIV-1 central single-stranded gap is defined by the central copy of the PPT. We can infer from this observation that the central PPT is used as an additional initiation site for the synthesis of the plus strand of HIV-1 DNA.

The single-stranded gap is found exclusively on linear DNA molecules. Electrophoretic analysis of undigested and of *Pst*I- or *Bam*HI-digested unintegrated HIV-1 DNA showed an approximately equal proportion of linear and circular molecules in acutely HIV-infected MT4 cells. Indeed, after *Pst*I or *Bam*HI digestion, the intensity of the shortened product resulting from digestion of linear molecules was approximately equal to that of the 9-kb linearized circles, found in both *Bam*HI and *Pst*I digestion reactions. Of interest is that no band with a size similar to that of the full-length linear molecules (corresponding to two-LTR linearized circles) could be observed in these reactions. This means that the vast majority of circular molecules in the acutely HIV-1-infected MT4 system have only one LTR.

Several lines of evidence indicate that circular molecules do not carry a single-stranded gap. First, the closed circles by definition cannot be gapped, although they are highly sensitive to nuclease S1 digestion (19). Second, the S1 digestion kinetics experiment (Fig. 2) shows that the 9-kb product corresponding to the linearized one-LTR circles was still present, even after 1 h of treatment with nuclease S1, when native linear molecules had almost completely disappeared. Third, after digestion with *Pst*I and *Bam*HI, which cut only once in the HIV-1_{bru} genome, nuclease S1 treatment did not generate products of a size compatible with fragments released from gapped circular molecules. For example, the *Bam*HI-nuclease S1 double digestion failed to release a 5.5-kb fragment, which would span from the *Bam*HI site to the gap in a circular one-LTR molecule and would be reactive with the 5′ probe. Similarly, the *Pst*I-nuclease S1 double digestion did not release any 3′-probe-reactive product of 5.7 kb, which would be the distance between the gap site and the *Pst*I site in a circular one-LTR molecule. Overall, the products observed in the double digests were of a size compatible only with DNA fragments released from native, linear two-LTR molecules.

Finally, we have observed that the proportion of circular versus linear molecules could vary with the source of the analyzed DNA. Figure 5 shows a comparison of nuclease S1 treatment of low-molecular-weight DNA from MT4 cells harvested 2 days after HIV-1 infection (lanes 1 to 4) and from CEM cells harvested 9 days after infection (lanes 5 to 8). In the CEM cells, the proportion of linear molecules was low, as shown by the lower proportion of 9.5-kb band than of open and closed circles on undigested lane 5. Another sign of the low proportion of linear molecules is visible on lane 7, where the intensity of the 1.2-kb fragment released by *Bam*HI is weaker than that of the 9-kb linearized circles. Coincidentally, the amount of subgenomic product released by nuclease S1 digestion of the same DNA samples was low. In the MT4 cells, in which more linear molecules were found, the quantity of subgenomic S1 digestion product was proportionally much higher than in the CEM cells.

DISCUSSION

We have observed that HIV-1 unintegrated linear DNA molecules, which accumulate in acutely infected CD4+ lymphoid cells, carry a short, unique, single-stranded region at the center of the genome. Both ends of this single-

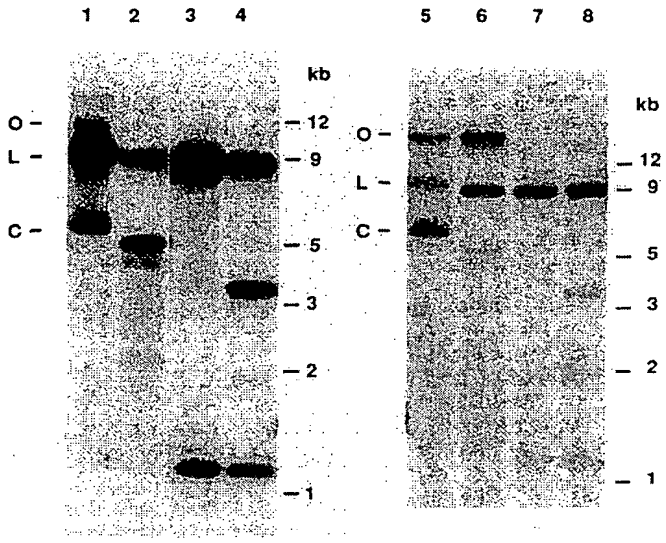

FIG. 5. Nuclease S1 sensitivity of HIV-1 unintegrated DNA from two different cell cultures. Lanes: 1 to 4, low-molecular-weight DNA from MT4 cells harvested 2 days after infection; 5 to 8, low-molecular-weight DNA from CEM cells harvested 9 days after infection. Unintegrated HIV-1 DNA was analysed by nuclease S1 treatment either with no previous restriction enzyme digestion (lanes 1, 2, 5, and 6) or after digestion with *Bam*HI (lanes 3, 4, 7, and 8). Lanes 1, 3, 5, and 7, No nuclease S1 treatment; lanes 2, 4, 6, and 8, treatment with nuclease S1 (1.5 U/μg of DNA). Both Southern blots were hybridized to the 3′ probe shown in Fig. 3B. Both autoradiograms are overnight exposures.

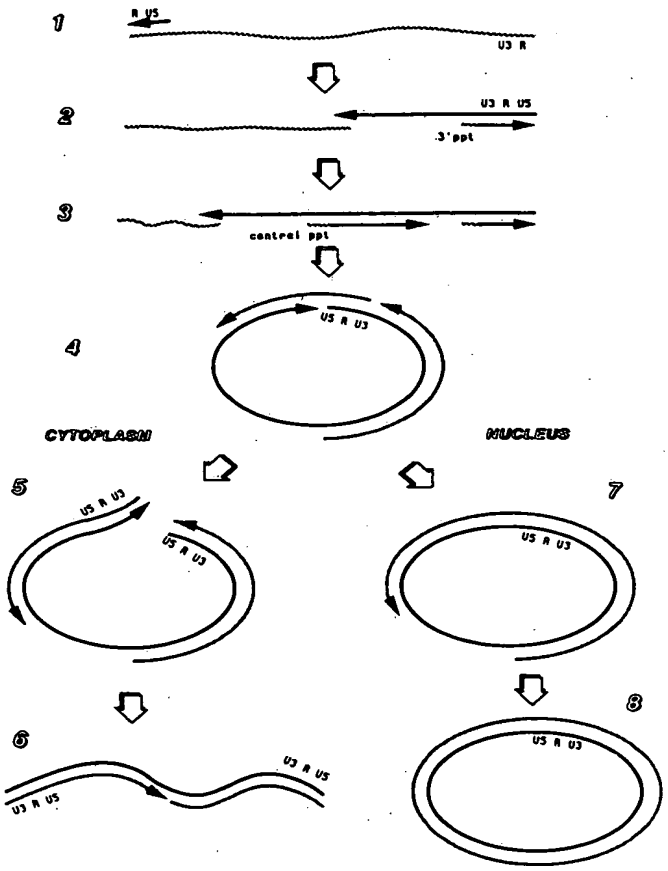

FIG. 6. Model for HIV reverse transcription. 1, Minus strong-stop synthesis; 2, first (minus-strand) template transfer and plus-strand strong-stop synthesis, initiated at the 3′ PPT; 3, progression of minus-strand synthesis and initiation of plus-strand synthesis at the central PPT; 4, second (plus-strand) template transfer; 5, formation of the LTRs by strand displacement and synthesis and progression of synthesis of the 5′ half of the plus strand; 6, linear gapped DNA molecule; 7, following step 4, ligation at both boundaries of the LTR, before strand displacement and synthesis can start, and progression of plus-strand synthesis; 8, ligation at the gap and formation of a one-LTR closed circle.

stranded gap are discrete. We have established that the 3′ boundary of this single-stranded element coincides with a PPT found at position 4800 in the HIV genome, an exact repeat of the structure normally found in all retroviruses next to the U3 region of the LTR (at position 9070 in the HIV-1 provirus). We have not determined the 5′ boundary of the gap and therefore cannot be certain of its size. However, analysis of the sizes of fragments released from nuclease S1-treated linear molecules following restriction enzyme digestions and corresponding to either the 3′ or the 5′ side of the gap suggests that this gap is short, probably less than 100 nucleotides in length.

In other retroviral models, the PPT has been shown to determine in vitro the initiation site of retroviral DNA plus-strand synthesis (20, 23, 27, 32). The DNA genomes of hepatitis B and cauliflower mosaic viruses, which are synthesized through reverse transcription of an RNA template, carry several single-stranded structures. In particular, there are two plus-strand discontinuities in the cauliflower mosaic virus genome that are defined by short PPTs which likely correspond to plus-strand initiation sites (25, 42). Our findings indicate that in HIV-1, and most likely also in other lentiviruses, the central PPT is used in vivo as an additional priming site for plus-strand synthesis. Indeed, primer extension experiments shown here reveal that the plus-strand DNA 3′ to the gap starts exactly at the last nucleotide of this PPT. This finding establishes that this structure determines precise and specific priming of plus-strand DNA at a central position in the genome. Because we used *Taq* polymerase and not reverse transcriptase in the primer extension experiments, we could not conclude whether the RNA primer corresponding to the central PPT remained attached to the nascent DNA strand, as described for other retroviruses at the 3′ PPT (7, 26).

It has been shown in avian retroviruses that plus-strand synthesis can start at sites distinct from the 3′ PPT, resulting in plus-strand discontinuities (16, 18). However, these initiation sites do not seem to be unique or well defined, and they do not result in a discrete single-stranded structure comparable to what we describe here. It is likely that the resulting plus-strand segments are eliminated by strand displacement events, as was shown in mellitin-permeabilized virions (4). In HIV, such a strand displacement is likely to occur at the 3′ PPT, to generate the 5′ end of linear molecules (Fig. 6, steps 4 and 5), but seems not to occur at the central PPT. We cannot explain why the upstream limit of the gap remains discrete. The gapped linear molecules represent a defined species: they are full-length, double-stranded molecules on which synthesis of the 5′ half of the viral plus strand is

stopped near the initiation site of the 3′ half. It is possible, however, that similar to what is found in cauliflower mosaic virus (25), elongation of the upstream HIV plus strand engages in a brief strand displacement event. Further studies are needed to clarify this point.

As a whole, our findings support a model of retroviral DNA synthesis (Fig. 6) in which linear molecules require a strand displacement step for LTR synthesis (4, 26), whereas one-LTR circles are the results of ligation events. Indeed, closed circular DNA molecules, which are found exclusively in the nucleus (17, 31), could be generated by ligation at both boundaries of the LTR and at the gap (Fig. 6, steps 7 and 8), following the proposed "intrastrand" plus-strand template transfer (24). This, in turn, could result from early nuclear transport of uncompleted DNA molecules.

Finally, it remains to be understood why lentiviruses, unlike other retroviruses, have developed and conserved a repeat of the PPT at the center of the genome. The most likely explanation is that it allows progression of plus-strand synthesis before elongation of the minus strand is complete (Fig. 6, step 3), probably resulting in a gain of time in DNA synthesis. The precise location of the PPT repeat at the center of the genome supports this hypothesis: an additional plus-strand initiation at this site could allow its elongation to reach the 3′ PPT approximately at the same time minus-strand synthesis is completed, the latest being required for plus-strand template transfer (Fig. 6, step 4). The LTRs can then be synthesized as plus-strand synthesis is being completed in the 5′ half of the genome (Fig. 6, step 5). Since lentiviruses, which are not transforming viruses, rely essentially on reverse transcription for their propagation, this feature could constitute an evolutionary advantage. The initiation of HIV plus-strand synthesis at the center of the genome is also interesting in view of recent observations that in unstimulated normal human lymphocytes, synthesis of the HIV minus strand appears to be arrested approximately halfway along the genomic RNA template (44). Dependence of full-length DNA synthesis on cell growth had also been described for avian viruses (9, 40). If the described stop in HIV minus-strand synthesis is located beyond the central PPT, it may allow early synthesis of a plus strand covering the whole 3′ half of the genome and may direct faster completion of double-stranded full-length DNA molecules upon further lymphocyte activation.

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